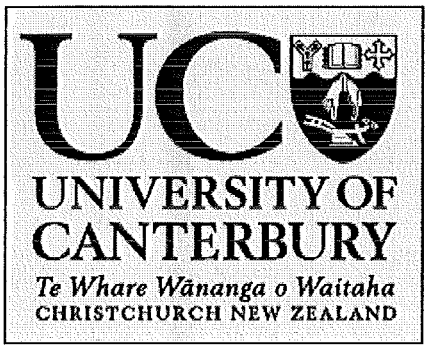
ENZYMATIC HYDROLYSIS OF OFFICE PAPER AND NEWSPAPER TO REDUCING SUGARS

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Engineering in Chemical and Process Engineering



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ABSTRACT

Abundant lignocellulosic biomass from municipal solid waste (MSW) is comprised of 40 % waste paper, which can be utilised to produce bioethanol, a renewable energy resource to reduce fossil fuel use. Eventhough lignocellulosic biomass provides a low cost resource, it is very difficult to convert into bioethanol and this results in the cost of bioethanol production being commercially unfeasible. Production of bioethanol from lignocellulosic biomass consists of two steps. The first step converts the biomass to fermentable sugars by hydrolysis. This is followed by microbial fermentation of the sugars to bioethanol. The objective of this study was to investigate hydrolysis of two types of waste paper, namely, office paper and newspaper, to produce fermentable sugars using the enzyme cellulase.

Enzymatic hydrolysis of waste paper is affected by numerous factors such as reaction time, enzyme/paper ratio, pretreatment and surfactant addition. A type of statistical design, i.e. Low Cost Response Surface Method (LCRSM) was used to study the main and interactive effects among the four significant factors of reaction time (4-20 h), enzyme/paper ratio (2-18 %), H_3PO_4 pretreatment (0-8 g/L), surfactant concentration (0-8 g/L for office paper and 0-12 g/L for newspaper) on sugar production efficiency. This method was proposed because it is comparable to other conventional experimental design methods with much lesser time, cost and effort in optimising the four variables to give the maximum sugar yield. The optimum conditions for office paper were obtained as follows: reaction time = 20 hours, enzyme/paper ratio = 18 %, H_3PO_4 = 8 g/L, surfactant = 4.2 g/L and predicted sugar yield = 86.6 %. For newspaper, the optimum conditions were: reaction time = 20 hours, enzyme/paper ratio = 18 %, H_3PO_4 = 8 g/L, surfactant = 6.8 g/L and predicted sugar yield = 18 %.The predicted sugar yields obtained with a commercial software package are similar to those predicted using Genetic Algorithms (GAs) and in good agreement with experimental sugar yields of 82.2 % and 17.13 % measured under the predicted optimum conditions for office paper and newspaper respectively.

Reaction time is a dominant factor as longer time periods are required for efficient interaction between cellulase and cellulose. Enzyme/paper ratio is another important factor because cellulase breaks down cellulose into reducing sugars. However, the rate of reaction is influenced by the structural features of cellulose. The structural features that govern the susceptibility of cellulose to enzymatic attack include: (1) the amount of lignin content, (2) the degree of crystalline portion, (3) moisture content of cellulose and (4) the accessible areas between the

microfibrils for cellulase molecules to penetrate through. Pretreatment has to be applied prior to hydrolysis. H_3PO_4 pretreatment was not so effective with substrate containing lignin, but is able to reduce the level of crystallinity of cellulose and further increase the accessible area. Therefore, both substrates experienced different extent of saccharification yield. Surfactant addition depends on the amount of lignin exposed to the environment solution surface and works effectively in preventing unspecific binding of cellulose onto lignin surfaces.

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1 INTRODUCTION

1.1 Biomass: A Valuable Resource

Nowadays, the standard of living around the world has vastly improved, thanks to technological advancements, which indirectly require intense energy utilisation to keep pace with our changing requirements. Our current primary energy source is fossil fuels, which caters for electricity generation, transportation, heating purposes and other uses. Unfortunately, fossil fuels are not a renewable energy resource and eventually, we will face a shortage of supply on this valuable source. As humans continue to make full use of the benefits of fossil fuels to create energy, more and more carbon dioxide (CO_2) and other harmful gases, such as carbon monoxide (CO) are being released to the environment. In the long term, this results in extensive damage to the atmosphere and future generations would never be able to experience the good quality of life as experienced by us.

The replacement of fossil fuels with renewable resources, such as biomass would provide the most promising solution to the major problems mentioned previously. Biomass is also known as lignocellulosics, which are comprised of three major components: cellulose, hemicellulose and lignin. Biomass can be converted to bioethanol via a two step process: the substrate is first hydrolysed using cellulase to form reducing sugars, followed by an addition of yeasts or bacteria to ferment these sugars into bioethanol. Utilisation of biomass for production of bioethanol is not a new technology. An abundant source of raw materials such as sugar cane, sugar beet, corn, oil seeds largely produced in the US and Brazil are employed for energy production. In the US alone for example, bioethanol continues to be the third largest use of corn, behind only feed and exports (RFA, 2004). Bioethanol can be used by blending with regular fuel. All cars with a catalyst can be run on a mixture of 90 % gasoline and 10 % bioethanol, while modern cars can safely use mixtures containing up to 20 % bioethanol (Galbe and Zacchi, 2002). However, the major use of bioethanol these days is as an oxygenated fuel additive, where the octane number in bioethanol is higher than gasoline and thus reduces the need to use toxic additives such as benzene (Wyman, 1996). Furthermore, bioethanol also provides oxygen for the fuel, which leads to a reduction of tailpipe emissions of CO and unburned hydrocarbons that are partly to blame for polluting our environment.

However, crop availability for sourcing bioethanol feedstock is a major issue. Crop growth is very much dependent on climate and environmental factors, cropping practice, culture type and nature of the local technology (Claassen, *et al.*, 1999). As a result, some societies may be reluctant to even use crop residues if they believe that somehow the food supply is inadequate. With this reason, other means of lignocellulosic biomass were proposed to support the large production of fuels and chemicals. These include forestry residues (e.g. mill wastes), herbaceous (e.g. leaves), woody (e.g. pine) and municipal solid waste (e.g. waste paper) or even animal manure.

While most cellulose used is present in tree or plant forms, immense amounts of cellulose already exist in cellulosic wastes, such as municipal solid waste (MSW). MSW typically consists of household waste and some fractions of industrial waste, with an average of 80 % being biodegradable (van Wyk, 2001). Waste paper products (e.g. office paper and newspaper) comprise the largest portion of MSW, with an average composition of 40 %. These lignocellulosic wastes mostly end up in landfills, are incinerated or for recycling purposes. Landfilling is the worst method to deal with waste papers as it requires a vast amount of space and poses health risks through the generation of greenhouse gases. Incineration does save landfill space, but produces combustion products that may be hazardous to health. Recycling is one method that has been promoted heavily in countries worldwide. Although recycled papers have been applied with great success, the recycling process weakens the fiber strength resulting in low paper quality. As a consequence, paper can only be recycled around 5-7 times before the fibers become too short. Other variables also affect the customers' demand such as greyish coloured papers produced due to incomplete removal of ink during the recycling process. Eventually, these recycled papers would still end up in landfills or are incinerated.

Waste papers could be utilised as a renewable resource for bioethanol development, as waste paper usage allows a reduction in pollution and the use of fossil fuels. As previously mentioned, the hydrolysis of cellulose to glucose can be catalysed by cellulase and then further fermented to form bioethanol. The overall process costs are far too expensive and the industry can only survive with government funding. Biofuel options are still incompatible with petrol or diesel as the lowest cost for bioethanol from Brazilian sugar cane is about 40 % more expensive than gasoline (DfT, 2003). The cost of cellulase is much too high and sugar yield is extremely low. Needless to say, fermentation of sugar to bioethanol is too low and is therefore not economically feasible. However, recent advances in research in this area of interest have shown ways to

optimise and improve the efficiency of sugar production to bring the enzymatic hydrolysis process closer to commercial viability.

1.2 Enzymatic Hydrolysis of Office Paper & Newspaper

Cellulose is a major component in paper material, constituting 85-99 % of office paper and 40-55 % of newspaper (Sun and Cheng, 2002). Using enzymatic hydrolysis process, cellulose in paper can be converted to sugar, which can then be further fermented to produce bioethanol and other value-added products.

Enzymatic hydrolysis uses cellulase enzyme extracted from fungi. Cellulase from two particular fungi, *Trichoderma reesei* and *Penicillium funiculosum*, has been the most extensively studied for hydrolysis of office paper and newspaper. Cellulase from *T. reesei* exhibits high glucanase activity and yields more sugar when used on office paper compared to cellulase from *P. funiculosum* (van Wyk, 1998). On the other hand, cellulase from *P. funiculosum* shows higher β-glucosidase activity and gives higher yield when used on newspaper (van Wyk, 1999a).

When used in combination, synergism between cellulase from *T. reesei* and *P. funiculosum* resulted in an increase in activity as compared to using a single type of cellulase alone. van Wyk (1999a) also found that the optimum ratio of the two cellulase is 1:1, which can be attributed to cross supplementation of cellulase components from the two fungi.

The degree of susceptibility and hydrolysis effectiveness of the paper to cellulase action depends on the paper structural features. The layers of cellulose molecules in paper exhibit different degrees of crystalline and amorphous regions. Caulfield and Moore (1974) indicated that the amorphous portion is hydrolysed at about twice the rate of the crystalline portion. Furthermore, the presence of lignin, 0-15 % in office paper and 18-30 % in newspaper, forms a seal around cellulose microfibrils that prevents it from being contacted by cellulase.

Although enzymatic hydrolysis shows promises, at the moment the process is still too slow with very low yield, making it uneconomical for larger scale operation. Two factors that relate to the economic potential, the enzyme/paper ratio and reaction time, can be improved by subjecting the paper to pretreatment prior to the hydrolysis and also by adding surfactant to the enzyme-

substrate mixture during the hydrolysis. The amount of these chemicals, for pretreatment and as surfactant, needs to be considered as it also affects the cost.

1.2.1 Enzyme-to-Paper Ratio

The ratio of total enzyme to the total substrate indicates the efficiency of the cellulase action. Paper conversion to sugar increases as more cellulase is used. This would however also increase the cost of the process. Furthermore, cellulase deactivates during the hydrolysis process (Palonen *et al.*, 2004; Lee *et al.*, 1982), making recovery of useful enzyme limited.

1.2.2 Reaction Time

Reaction time is measured as the duration taken to reach an acceptable level of cellulose conversion. The rate of hydrolysis typically slows down after a certain period and further decays towards the end when no more hydrolysis is expected. While prolonging the reaction time may increase the cellulose conversion, this will also increase the production cost. Shorter reaction time is preferable as it indicates higher rate of hydrolysis and possibly increased yield.

1.2.3 Pretreatment

Pretreatment enhances hydrolysis by disrupting the crystalline structure of cellulose and its close association with lignin, making the paper more vulnerable to hydrolysis. A variety of different pretreatment methods for office paper and newspaper had been identified. This includes, among others: ammonia freeze explosion process (Sun and Cheng, 2002) and ultrasound (Li *et al.*, 2004). All these processes can enhance cellulose conversion. Phosphoric acid (H_3PO_4) pretreatment which had not been used in newspaper and office paper will be expected to heighten the cellulose digestibility.

1.2.4 Surfactant Addition

Several studies have discovered that surfactant addition can increase cellulose conversion by reducing the irreversible binding of cellulase onto lignocellulosic materials (Alkasrawi *et al.*, 2003; Eriksson *et al.*, 2002; Wu and Ju, 1998). Castanon and Wilke (1981) found that the hydrolysis of newspaper was increased by 14 % after 48 hours and more than twice as much

cellulase recovered with the addition of non-ionic surfactant Tween 80 or chemically known as *polyoxyethylenesorbitan monoleate*. Although these studies are not based on office paper as substrate, similar effect is anticipated with office paper.

1.3 Research Objectives

To the author's best knowledge, no studies have yet examined the interactions of all the four factors mentioned above. Most studies had been done to optimise the hydrolysis process by changing only one of the factors at a time. Other potentially important factors were not studied together, usually because of experimental budget constraints. Hence, the main aim of this study was to examine the effect of the four factors, i.e. enzyme/paper ratio, reaction time, H_3PO_4 pretreatment and non-ionic surfactant Tween 80 requirement on the enzymatic hydrolysis of waste paper to reducing sugars.

To reduce the number of experiments, response surface methodology (RSM) is commonly used to identify and optimise the significant factors. The particular RSM chosen for this study is the Low Cost Response Surface Methods (LCRSMs) first proposed by Allen and Yu (2002). Other popular RSMs such as Box-Behnken Design (BBD) (Hari Krishna and Chowdary, 2000; Tengborg *et al.*, 2001) and Central Composite Design (CCD) (Wen *et al.*, 2004) are not chosen as the number of experimental runs required by these methods is still considered too large and time-consuming. The five-level, four-factor fractional design using LCRSM requires only half the number of runs required by BBD and CCD with similar modelling error expected. LCRSM is based on linear regression and can be applied using statistical software such as *Design Expert Version 6*. A quadratic polynomial model will be built using the software to find optimum conditions for all the four factors.

The performance of the LCRSM will be compared to a genetic algorithm. The purpose is to find out whether LCRSM has the same performance obtained using a genetic algorithm. Genetic algorithms are optimisation techniques useful in functions whose nonlinearity makes an analytical optimisation impossible. The calculations were carried out using Matlab 6.

2 LIGNOCELLULOSICS AND HYDROLYSIS OF CELLULOSE

This chapter gives an overview of the structure and chemical composition of lignocellulosics, properties of enzymes and their mode of action, factors limiting enzymatic hydrolysis of cellulose, various pretreatment methods and surfactant addition to improve sugar yield.

2.1 Structural Features of Lignocellulosic Materials

Lignocellulosic materials are comprised of three principal components: cellulose, hemicellulose and lignin (see **Figure 2.1**). The cellulose of these materials can be hydrolysed to produce fermentable sugars. Native lignocellulosics is very resistant to enzymatic hydrolysis, and this is primarily due to their structural features. These include (1) cellulose present in lignocellulosics consists of a highly resistant crystalline structure and amorphous components (2) hemicellulose binds with cellulose to form microfibrils and also cross-links with lignin to provide structural strength and (3) lignin is the most recalcitrant component in lignocellulosics and forms a physical barrier surrounding the cellulose. Amorphous components are very susceptible towards enzymatic attack, but they are well-protected by the lignin seal. The differences in chemical composition of assorted types of lignocellulosic materials are shown in **Table 2.1**. In general, these materials can be regarded as being composed of 50 % cellulose, 25 % hemicellulose and 25 % lignin. The susceptibility of lignocellulosic materials can be improved by means of pretreatments to expose the cellulose, thereby allowing the enzymes to penetrate and hydrolyse the cellulose to reducing sugars (Yang and Wyman, 2004; Imai *et al.*, 2004, Mooney *et al.*, 1998; Nazhad *et al.*, 1995; Lin *et al.*, 1985).

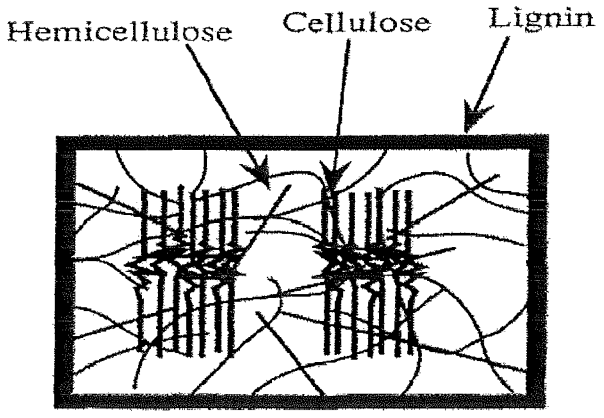


Figure 2.1 Schematic representation of the main components in inedible plant material (Adapted from Kohlmann et al., 1996)

Table 2.1 Chemical composition of lignocellulosic materials

Lignocellulosic	Cellulose	Hemicellulose	Lignin	Reference
Spruce wood	46.1	24.6	27.8	Cowling and Kirk, 1976
Pine wood	37.7	11.6	27.5	Hayn *et al.*, 1993
Poplar wood	49.9	25.1	18.1	Wiselogel *et al.*, 1996
Softwood (pine & spruce)	43.0-45.0	20.0-23.0	28.0	Galbe and Zacchi, 2002
Corn stalk	33.5	32.6	11.1	Ladisch, 1989
Corn stover	36.4	19.6	16.6	Wiselogel *et al.*, 1996
Bagasse	38.0	34.0	11.0	Ladisch, 1989
Wheat straw	36.0	31.0	7.0	Felby *et al.*, 2003
Waste cellulose fibers	75.2	6.8	<1.0	Nikolov *et al.*, 2000
Leaves	15.0-20.0	80.0-85.0	20.0	Sun and Cheng, 2002
Grasses	22.0-40.0	35.0-50.0	10.0-30.0	Sun and Cheng, 2002
Kraft pulp	76.9	13.4	3.4	Boussaid and Saddler, 1999
Paper	85.0-99.0	0.0	0.0-15.0	Sun and Cheng, 2002
Newspaper	40.0-55.0	25.0-40.0	18.0-30.0	Sun and Cheng, 2002
Newspaper	61.0	16.0	23.0	Van Wyk *et al.*, 1999b

2.1.1 Cellulose

Cellulose is the major constituent of plant cell walls of wood and other plant parts, comprising about 50 % of the total wood. Cellulose is a long chain of individual glucose molecules, linked together by β-1-4 glycosidic linkages. The successive glucose residues are rotated by 180° relative to each other and hence, the replicating unit in the cellulase chain is known as cellobiose (see **Figure 2.2 (a)**). The number of chain units or degree of polymerisation (DP) varies mostly between 500-10,000 glucose units, depending on the source (Fan *et al.*, 1987).

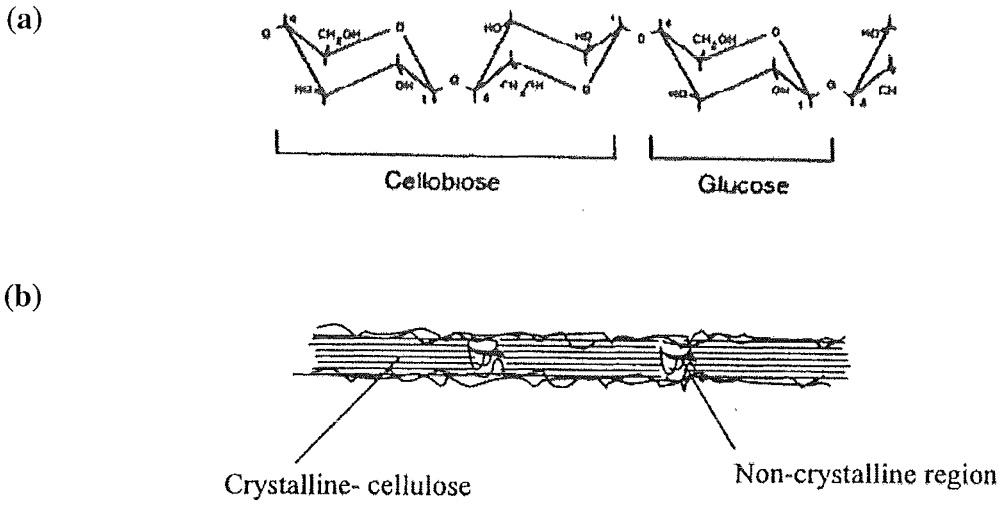


Figure 2.2 (a) Representation of a single cellulose chain. (b) Schematic diagram of a longitudinal section through cellulose microfibrils illustrating the crystalline and non-crystalline (amorphous) region. (Adapted from Quentin et al., 2003)

The cellulose chain has OH-groups at both ends and is stabilised by strong hydrogen bonds along the direction of the chain. The chains held together by the hydrogen bonds are packed together to form a highly crystalline material that is recalcitrant to enzymatic hydrolysis in native cellulose (Mansfield *et al.*, 1999; Gregg and Saddler, 1996). Native cellulose also contains amorphous cellulose in a less-ordered manner (see **Figure 2.2 (b)**) that has much less resistance as compared to crystalline regions (Mansfield *et al.*, 1999). In addition, the presence of other components such as hemicelluloses, together with cellulose, makes the plant cell wall a very poorly accessible substrate. Several studies had been done to survey the structure of cellulose and are still currently under intense study (Junior, 2000; Kataoka and Kondo, 1998; Mansfield *et al.*, 1997).

2.1.2 Hemicellulose

Hemicelluloses can be divided into three main groups, i.e. xylans, mannans and galactans. Hemicelluloses are closely associated with cellulose fibrils to form microfibrils that can enhance the stability of the cell wall. They can also cross-link with lignin to provide rigidity and strength to the wall. The most common hemicellulose component found in hardwoods is xylan, whereas in softwoods, mannan is the most abundant type (Quentin *et al.*, 2003). According to Kohlmann *et al.* (1996), solubilising hemicelluloses can significantly increase the pore volume, thereby increasing substrate susceptibility towards enzyme accessibility and hydrolysis.

2.1.3 Lignin

Lignin is probably the most complex, comprises of hydrophobic surface, which is cross-linked to each other with a variety of different chemical bonds. Lignins are polymers derived from coniferyl-, sinapyl- and p-coumaryl-alcohol and the compositions vary widely with the plant source. Lignins are mostly found as an integral part of the plant cell wall, embedded in the matrix of the cellulose and hemicellulose. Linking takes place by different chemical bonds and this results in highly hydrophobic polymers. This makes lignin the most recalcitrant component of the plant cell wall and capable of resisting to mechanical stress.

Recent studies indicated that not all lignins are homogeneous in structure. Lignins consist of amorphous regions and structured forms such as oblong particles and globules (Novikova *et al.*, 2002). However, lignin in the outer layer of the plant cell wall is not amorphous as it needs to provide structural rigidity to the plant. Phenyl rings of softwood lignin have been shown to be aligned preferentially in the plane of the cell wall (Atalla and Agarwal, 1985). According to Houtman and Atalla (1995), both the chemical and three-dimensional structures of lignin are strongly influenced by the polysaccharide matrix. Molecular dynamic simulations demonstrated that the hydroxyl and methoxyl groups in lignin precursors and oligomers may interact with cellulose, although lignin is hydrophobic in nature (Houtman and Atalla, 1995).

2.2 Hydrolysis of Lignocellulosic Materials

2.2.1 Acid Hydrolysis

Acid hydrolysis processes have been used in the past to convert lignocellulosic materials into sugars, particularly in the former Soviet Union, Japan and Brazil (Sheehan and Himmel, 1999). The history of sugar production by acid hydrolysis dates back to the year 1819 (Sheehan and Himmel, 1999). Since then, numerous studies have shown that acid hydrolysis can achieve high yield of sugar production from lignocellulosics. There are two common types of acid hydrolysis processes, i.e. concentrated and dilute acid hydrolysis.

Dilute acid hydrolysis of lignocellulosics has been carried out mainly with sulphuric acid. The main advantages of using diluted sulphuric acid are its relatively low acid consumption and low plant capital cost. Moreover, it is easy to separate dilute sulphuric acid from the hydrolysis medium. However, the disadvantage is that the sugar yield is relatively low. In order to increase

the sugar concentration, large amounts of acid are needed and this causes problems with equipment corrosion and requires high acid recovery. Besides, high temperature is also required to improve hydrolysis rate. The maximum sugar yield was obtained at a high temperature and short residence time, but the conversion rate was only 55 % (Fan *et al.*, 1987).

Concentrated acid hydrolysis processes are operated at low temperature, short residence time and a high amount of acid can achieve very high sugar yield of 97 %, compared to dilute acid process (Keller, 1996). The concentrated acid disrupts the hydrogen bonding between the cellulose chain and solubilising hemicellulose to form sugar polymers (Sheehan and Himmel, 1999). Still, high amounts of concentrated acid usage can lead to equipment corrosion problems and high-energy demanding acid recovery. As mentioned earlier, concentrated acid processes require large amounts of acid and the recovery of the concentrated acid is not cost-effective. Moreover, neutralisation of acid produces large amount of gypsum (Keller, 1996).

Recently, enzymatic hydrolysis has been proposed as an alternative to acid hydrolysis as it offers highly efficient conversion with few or no by-product problems. An overview of enzymatic hydrolysis is given in the next section.

2.2.2 Enzymatic Hydrolysis

Unlike acid-based processes, enzymes are the relative newcomers with respect to biomass-to-ethanol conversion. The search for biological causes of cellulose hydrolysis began during World War II when the American armies put up a basic research program to understand the causes of deterioration of military clothing and equipment in the jungles of the South Pacific, a problem that was causing mayhem with cargo shipments during the war. The campaign resulted in the formation of a research center called Army Natick Laboratory (Sheehan and Himmel, 1999). Among the celluloytic fungi and bacterial cellulase that have been identified, *Trichoderma viride*, now known as *Trichoderma reesei,* is the most widely researched of all potent enzyme-producing fungi conducted in the laboratory (Himmel *et al.*, 1996) to prevent enzymatic hydrolysis attack on cellulose.

The usage of enzymes to improve hydrolysis on lignocellulosics did not occur until the early 1960s, when sugars were recognised as a possible energy product, recalling similar opinions expressed by those researchers in the earlier days of acid hydrolysis processes. As a result of intense study, enzymes have now been recognised as playing a key role in producing sugars from

lignocellulosics. The process is often performed at an optimum temperature of 50 °C and a pH of 4.8 (Ingram and Doran, 1995) utilising enzymes as catalysts, hence providing a very specific conversion of cellulose. In addition, it is also believed that enzymes have a higher potential to produce higher sugar yields and less or no by-product formation as experienced by acid hydrolysis (Keller, 1996). Among the lignocellulosic materials that are most commonly considered for conversion are wood, agricultural wastes, grasses and the paper fraction of municipal solid waste.

2.2.2.1 Hydrolytic Action of Cellulase

The mechanism of cellulase (a complex system of enzymes that work synergistically to attack cellulose) was first proposed by Reese and his co-workers in the 1950s (Gan *et al.*, 2003). According to their hypothesis, the conversion of cellulose to sugar by cellulase was depicted as a two-step process. The C_1 component (exocellulase) acts to disaggregate or 'activate' the cellulose chains, followed by C_x (endocellulase), which breaks down the cellulose to sugar (Sheehan and Himmel, 1999).

However, Wood *et al.* (1997) asserted that the C_1 component was unable to attack crystalline cellulose alone and can only be accomplished through synergistic action with the separate C_x and β-glucosidase components (Fan *et al.*, 1987). Nonetheless, the C_1 component was able to break down the cellulose chain to cellobiose (a reducing sugar that consists of glucose units). Berghem and Petterson (1973) later concluded that the C_1 component is indeed a β-1,4-glucan cellobiohydrolase.

In general, the hydrolytic action of cellulase is accomplished by three major classes of enzymes (see **Figure 2.3**):

- Endo-1-4-β-glucanases randomly attack soluble and insoluble glucose chains;
- Exo-1,4-β-D-glucanases liberate glucose monomers from the end of the cellulose chain to form cellobiose;
- β-glucosidase (cellobiase) acts to release glucose units from cellobiose and soluble cellodextrins.

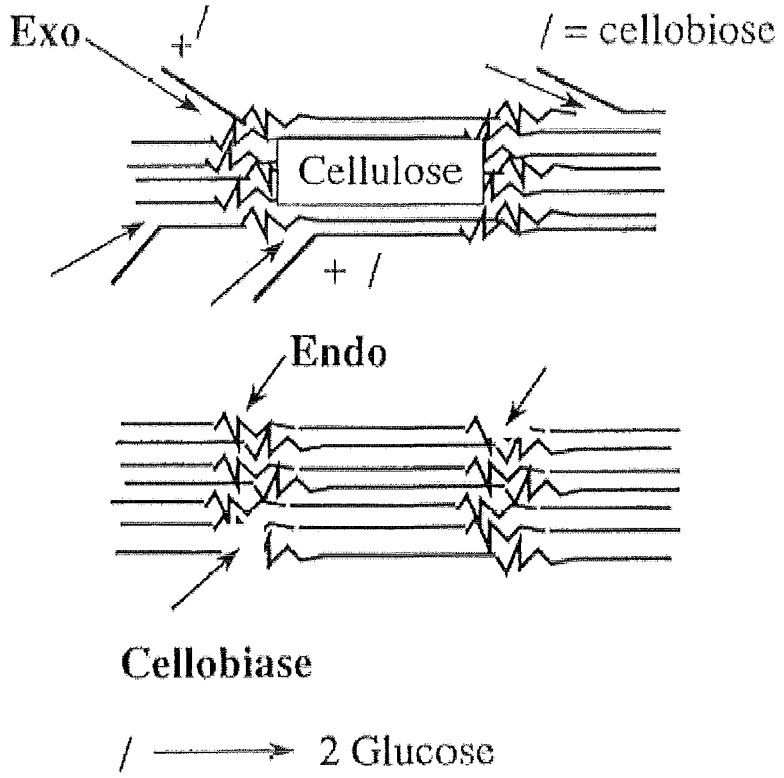


Figure 2.3 Schematic of cellulase action on cellulose showing endoglucanases, exoglucanases and cellobiase activity (Adapted from Kohlmann et al., 1996)

Fan *et al.* (1987) reported that synergism was a function of the ratio of individual enzyme components and maximum sugar yield can be achieved as total enzyme concentration is increased. Gregg and Saddler (1996) also agreed that the efficiency of enzymatic hydrolysis of cellulose not only depends on the synergism of cellulase components, but also on the optimum ratio of endoglucanase to exoglucanase. Despite the fact that the degree of synergism is at the maximum level as the cellulase concentration is increased, it still declines at a certain point due to saturation of the adsorption sites with cellobiohydrolase, hence decreasing the generation of chain ends by endoglucanase (Gan *et al.*, 2003; Converse and Optekar, 1993)

2.2.2.2 Hydrolytic Enzymes of *Trichoderma Reesei*

The cellulase complex of *T. reesei* has been the most widely studied. *T. reesei* is a filamentous fungus and can efficiently degrade cellulose and hemicellulose to reducing sugars. Cellulase secreted by the fungus consists of three major enzyme components: endoglucanases (EC 3.2.1.4), exocellobiohydrolases or exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Palonen *et al.*, 2004; Ramos *et al.*, 1999). These enzymes have distinct activities and occur in

multiple forms. Endoglucanases hydrolyse the internal bonds in the cellulose chain and attack mainly on the amorphous parts of the cellulose, therefore increasing the specific area for exoglucanases catalysis (Ramos *et al.*, 1999). Exoglucanases hydrolyse from the chain ends and predominantly produce cellobiose. There are at least five endoglucanases that have been identified, namely EG I-V and EC 3.2.1.4 (Palonen *et al.*, 2004). All *T.reesei* cellulase, except EG III, as well as many other cellulase from other microorganisms have a two domain structure consisting of a catalytic domain (CD) and a cellulose binding domain (CBD), which are bound together by a flexible linker (Palonen *et al.*, 2004; Gilkes *et al.*, 1991). On the other hand, cellobiohydrolases (CBH) I and II are three-dimensional structure enzymes and they have been shown to act synergistically during the enzymatic hydrolysis of cellulose (Palonen *et al.*, 2004; Linder and Teeri, 1997). The structural difference between endoglucanases and exoglucanases is that endoglucanases have an open active site that enables action in the middle of the glucan chain, whereas exoglucanases have a tunnel-shaped active site that can hydrolyse only chain ends (Teeri, 1997).

The ability of cellobiohydrolases to degrade crystalline cellulose decreases when the CBD is absent (Linder and Teeri, 1997). It has been observed that CBD increases the enzyme concentration on the surface of a solid structure of cellulose substrate and may lead to solubilisation of individual glucan chains from the cellulose surface (Teeri, 1997). However, the CBD may also bind specifically and non-specifically to the hydrophobic substrate surface at higher enzyme concentration (Linder and Teeri, 1997). The interactions are normally non-covalent, i.e. hydrogen bonding, electrostatic or hydrophobic interactions (Palonen *et al.*, 2004). The hydrophobic residues of cellulase may also lead to binding to the hydrophobic lignin surface.

Another major type of enzyme required for complete cellulose hydrolysis is β-glucosidases, which hydrolyse short cello-oligosaccharides to glucose. The β-glucosidases concentration in *T.reesei* is low and addition of this enzyme is required to prevent end-product inhibition.

Although enzymatic hydrolysis by the cellulase of *T.reesei* provides an environmentally sound system to convert cellulose to sugar, the overall conversion is extremely slow, at less than 20 % of theoretical maximum (Kim and Hong, 2001). Increasing cellulase concentration can improve sugar yield to a certain extent, but this would increase the overall process cost (Eriksson *et al.*, 2002; Wood *et al.*, 1997; Gregg and Saddler, 1996; Ingram and Doran, 1995; Fan *et al.*, 1987). Therefore, improvements on cellulase effectiveness are essential in order to decrease cellulase

consumption to an acceptable level and further increase sugar yield. Some of the factors that limit the hydrolytic action of cellulase are discussed in the next section.

2.3 Factors Limiting Enzymatic Hydrolysis

Enzymatic digestibility involved in the complete degradation of lignocellulosics is affected by a number of substrate structural factors. Even in the enzymatic hydrolysis of pure cellulose, a declining rate of sugar production is observed as the reaction proceeds. Reasons given for this decreasing hydrolysis rate are thought to be due to several factors such as cellulose crystallinity, surface area accessible to cellulase attack, cellulose protection by lignin or cellulase inactivation and unproductive binding onto lignin. However, the complete mechanism of cellulose hydrolysis cannot be fully resolved due to the heterogeneous nature of lignocellulosics and interference of other components such as lignin.

2.3.1 Crystallinity of Cellulose

Fan *et al.* (1987) estimated the proportion of crystalline or amorphous material in native cellulose to be in a range of 50-90 %. The degree of crystallinity was thought to play a major role in limiting hydrolysis (Lee and Fan, 1982; Fan *et al.*, 1980). It has been suggested that during enzymatic hydrolysis, the amorphous component of cellulose was hydrolysed first, leaving the more recalcitrant crystalline component unhydrolysed. Others supported the theory by suggesting that there was a significant increase in crystallinity during hydrolysis of cellulose with cellulase. They observed that during depletion of amorphous cellulose, the substrate becomes more crystalline, thereby offering an increased resistance to further hydrolysis (Koullas *et al.*, 1990; Fan *et al.*, 1987).

While lignocellulosics used to study the effect that crystallinity has hindered the hydrolysis rate were proven by some authors, others failed to demonstrate a positive relationship between crystallinity and rate of hydrolysis (Ramos *et al.*, 1993; Caulfield and Moore, 1974). Caulfield and Moore (1974) reported that ball milling increases the susceptibility of both amorphous and crystalline components of cellulose. In fact, cellulose crystallinity on hardwood and wheat straw was found to be higher after pretreatment and digestibility actually increased after pretreatment (Hsu, 1996). Likewise, steam pretreatment of lignocellulosics increases the crystallinity index of the substrate at the same time as it heightens the ease of hydrolysis of the substrate (Mansfield *et*

al., 1999). This contradicts the fact that the susceptibility of crystalline component is lower than that of the amorphous component. They concluded that the overall increase in digestibility is most likely due to an increase in accessible surface area (see **Section 2.3.2**).

2.3.2 Accessible Surface Area

The accessibility of lignocellulosics has been shown to play an essential role in the improvement of enzymatic hydrolysis (Mooney *et al.*, 1998; Grethlein, 1985; Lin *et al.*, 1985). The reaction between cellulose and cellulase is heterogeneous, and hence the hydrolysis rate is dependent on the amount of cellulose surface accessible to the enzyme molecules. In untreated cellulose, only a minor fraction of pores are accessible to cellulase. In the early years, many researchers did not consider accessible surface area as a crucial factor that affects the digestibility of native cellulose. Instead, they reported that crystallinity influences hydrolysis to a certain extent (Myerly *et al.*, 1981; Fan *et al.*, 1980). In later work, Grethlein (1985) showed that improvement of digestibility is related to the pore volume accessible to cellulase and claimed that crystallinity has no relationship to hydrolysis rate. A few studies indicated that drying of lignocellulosics and the consequence collapse of the cell wall capillaries and decrease in pore size reduces the effectiveness of enzymatic hydrolysis (Esteghlalian *et al.*, 2001; Wong *et al.*, 1988). Studies also showed the significant difference between softwood and hardwood efficiency is due to differences in terms of pore volume and ease of hydrolysis. It was reported that the pore volume of white pine is only half of the value obtained with a mixed substrate and that it was subsequently hydrolysed less efficiently (Mansfield *et al.*, 1999). The smaller pore volume of softwood is attributed to the presence of lignin in the pulp and lignin removal has a significant effect on enzyme digestibility (Mooney *et al.*, 1998). Nonetheless, accessible surface area may correlate to crystallinity, or to lignin protection, or both.

2.3.3 Presence of Lignin

It is widely recognised in literature that lignin content has a great impact on enzymatic hydrolysis (Yang and Wyman, 2004; Mansfield *et al.*, 1999; Mooney *et al.*, 1998; Vinzant *et al.*, 1997). Lignin, with partially crystalline cellulose existing in wood comprises one of nature's most biologically resistant materials. Intrinsically, lignin is capable of protecting cellulose from environmental exposure. Clearly, cellulase is prevented from degrading cellulose by the presence of lignin. It has been accounted that lignin removal increases the porosity of both kraft and sulfite pulps as it corresponds to the increased susceptibility of cellulose to hydrolysis (Mansfield

et al., 1999). Substantially higher enzymatic conversion of cellulose has been obtained from delignified pulp, containing 8.2 % lignin or kraft pulp with 4.4 % lignin, as compared to untreated pulp, containing 27.3 % lignin (Mooney *et al.*, 1998). Nevertheless, partial lignin removal, with a final lignin content of 32-36 % from steam pretreated softwood by alkaline NaOH pretreatment, has resulted in decreased hydrolysis yield (Wong *et al.*, 1988). Redeposition of unextracted lignin to the accessible surfaces was the reason given by Wong *et al.* (1988). In the case of wheat straw, the hydrolysis rate increased substantially up to 50 % with an increase in the extent of delignification. Beyond this, the hydrolysis rate only increased slightly, as described previously by Fan *et al.* (1987). Thus, it is apparent that delignification creates additional surface area, but the extent to which lignin adsorbs cellulase much depends on the nature of the substrate itself.

2.3.4 Adsorption of Cellulase to Cellulose

Efficient degradation of cellulose requires effective interaction between substrate and cellulase. It has been reported that cellulase interacts with cellulose surface through a cellulose-binding domain (CBD), in addition to a catalytic domain (CD). The overall binding efficiency of cellulase is greatly intensified by the presence of CBDs and the enhanced binding correlates with better activity towards crystalline cellulose (Aehle, 2004; Kormos *et al.*, 2000; Linder and Teeri, 1997; Gilkes *et al.*, 1992). It has been shown that the ability of cellobiohydrolases to degrade crystalline cellulose clearly diminishes when CBD is absent, but not on amorphous ones (Linder and Teeri, 1997). Even so, the exact role and action mechanism of CBDs is still a matter of speculation. Stahlberg *et al.* (1991) intimated that CBD improves the enzymatic activity of cellulase by enhancing the effective enzyme concentration merely onto lignocellulosics surface, but at the same time the binding via CBD can also lead to a population of unproductive bound cellulase. However, considering CBD function alone is not the key in determining the sugar conversion efficiency of lignocellulosics. This is because substrate heterogeneity with differing crystallinity and chemical composition may partly contribute to the observation of irreversible binding of cellulase and CBDs. Besides that, the binding of an intact cellulase can occur through either one or both domains, separately or simultaneously, and each different way of binding also has a different affinity (Linder and Teeri, 1997). Simultaneously, the domains of an intact cellulase are most likely to be bound by the cellulose surface that is known as a continuous array of overlapping binding sites, leading to irreversibility. It has also been suggested that catalytic and binding domains of different cellulase may have different preferred binding sites on the

cellulose surface and the dominating mode of binding may depend on each individual cellulase concentration (Stahlberg *et al.*, 1991). All these factors may lead to irreversible CBDs binding.

Several authors reported that presence of lignin can also have a significant effect on cellulase adsorption and it has been shown to irreversibly adsorb cellulase (Yang and Wyman, 2004; Boussaid and Saddler, 1999; Mooney *et al.*, 1998). It is apparent that high lignin substrate experienced incomplete hydrolysis even at high cellulase loading, indicating that cellulase binds to the lignin fraction of lignocellulosics. While the adsorbed cellulase remained associated with the residue, the recalcitrance of the residual substrate also restricted the release of the adsorbed cellulase back into the solution. In addition, Boussaid and Saddler (1999) found out that there was a proportional distribution of individual enzymes between soluble and insoluble phases of a refiner mechanical pulp (RMP) (30 % lignin), thus showing that unspecific adsorption of cellulase onto cellulose occurs within high lignin substrate.

It would be beneficial to be able to decrease non-specific adsorption of cellulase onto the lignocellulosics surfaces, making them more effective in enzymatic hydrolysis or even using lower enzyme concentration to achieve higher hydrolysis rate and sugar yields.

2.4 Pretreatment Prior to Enzymatic Hydrolysis

It has been known that the heterogeneous chemical reactions of cellulose are largely controlled by the highly ordered intra- and intermolecular packing of its crystalline regions. In addition, the rate-limiting substance that acts as a deterrent to substrate penetrability is lignin. Biodegradation of untreated native lignocellulosics is extremely low and this low rate and extent of conversion inhibit the development of an economically feasible process. To improve the hydrolysis rate, pretreatment is generally applied prior to enzymatic hydrolysis with minimal costs.

There are several desirable goals for pretreatment processes. However, in practice not all of them are achieved with any current pretreatment. Pretreatment methods can generally be grouped into three categories: physical, chemical and biological. Of the many pretreatment methods, some have been demonstrated to be effective in removing lignin, while others in disrupting the highly ordered crystalline region itself. The various pretreatment methods have been reviewed by Sun and Cheng (2002), Weil *et al.* (1994) and Fan *et al.* (1987). A summary of these methods and their advantages and drawbacks is given in the following sections.

2.4.1 Physical Pretreatments

2.4.1.1 Ball Milling

The ball milling method has been used to reduce cellulose crystallinity. Ball milling allows for a high slurry concentration, thereby reducing the reactor volume and thus, the capital cost. It was reported to be effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of substrate (Sun and Cheng, 2002), but produced least response on softwoods (Fan *et al.*, 1987). Fan *et al.* (1987) also described a high saccharification yield of 72.9 % for ball milled newspaper print in 48 hours. Although ball milling is an effective pretreatment method, it is time-consuming, energy-intensive and its expensive processing cost makes it impractical on large scale apparatus.

2.4.1.2 Steam

Steaming or steam explosion is one of the most investigated pretreatment methods for lignocellulosic materials. Steam explosion is generally initiated at a high temperature of 160-260 °C and a high pressure of 0.69-4.83 MPa for several seconds to a few minutes before exposing to atmospheric pressure (Walker and Wilson, 1991). The steam explosion method has the advantage of using 70 % less energy than conventional mechanical methods (e.g. ball milling) to achieve the same size reduction (Holtzapple *et al.*, 1989). The process causes hemicellulose solubilisation and lignin transformation due to its high temperature and pressure process, thus increasing the potential of cellulose hydrolysis. Grous *et al.* (1986) reported that 90 % efficiency of enzymatic hydrolysis for steam pretreated poplar chips could be achieved within 24 hours, as compared to only 15 % efficiency hydrolysis of untreated chips. Optimum hemicellulose solubilisation and hydrolysis can be achieved by either high temperature and short reaction time (270 °C, 1 min) or low temperature and long reaction time (190 °C, 10 min) (Sun and Cheng, 2002).

However, the pretreatment technique often suffers from incomplete destruction of a portion of xylan fraction that may be inhibitory to microorganisms used in downstream processes. Because of the formation of the inhibitory product, pretreated substrate needs to be washed with water to withdraw the inhibitory materials along with water-soluble hemicellulose. This will lead to an overall reduction in sugar yield.

2.4.1.3 Ammonia Fiber Explosion (AFEX)

The AFEX process is quite similar to steam explosion, where lignocellulosics are exposed to liquid ammonia at high temperature and pressure for a period of time, before the pressure is quickly reduced. AFEX typically operates at mild temperature (50-80 ^{o}C) and mild pressure (1.55 MPa). Various substrates including alfafa, wheat straw, softwood newspaper and rice straw have been tested to compare AFEX and steam pretreatment. It was discovered that the chemical composition after using AFEX alters slightly, or is similar to the original materials. Nonetheless, AFEX pretreatment works effectively on Bermuda grass (5 % lignin) and bagasse (15 % lignin), but not on newspaper (30 % lignin) and aspen chips (25 %). Hydrolysis yields of AFEX pretreated newspaper and aspen chips were reported to be below 50 % (Sun and Cheng, 2002; Weil *et al.*, 1994). It seems that AFEX pretreatment effectively pretreats agricultural residues and herbaceous crops, but not on substrates derived from wood (Galbe and Zacchi, 2002), although testing on woods has not been reported extensively.

2.4.1.4 Carbon Dioxide (CO_2) Explosion

Similar to steam and AFEX, CO_2 explosion is also used to pretreat lignocellulosics. It was hypothesised that once CO_2 is dissolved in water, it will form carbonic acid that would hydrolyse hemicellulose and cellulose as well as increase the accessible area of the substrate to enzymatic hydrolysis, thus increasing the reaction rate. Although Puri and Mamers (1983) demonstrated that the CO_2 explosion technique was effective, Dale and Moreira (1982) showed that it was less effective than AFEX. The sugar yield obtained during 24 hours of enzymatic hydrolysis was found to be low, compared to steam or AFEX. On the other hand, Zheng *et al.* (1998) carried out the CO_2 pretreatment process using repulping wastes of recycled paper, bagasse, recycled paper mix and Avicel (microcrystalline cellulose) as substrates and ascertained that CO_2 enhances the reaction up to 72.6 %, is cost-effective compared to AFEX and does not cause degradation of sugars such as those treated with steam explosion. A significant increase of final sugar yield was also obtained by Kim and Hong (2001) from enzymatic hydrolysis of pretreated aspen, at 85 % of the theoretical maximum. However, the overall sugar yield obtained for southern yellow pine was relatively low, at 27 % of the theoretical maximum. Still, the final sugar yield for pretreated pines was still higher in comparison with untreated pines. It was concluded that the CO_2 pretreatment method was not so effective for softwoods and high capital cost makes this form of pretreatment uneconomical on a large scale.

2.4.1.5 Ultrasound

Ultrasound was initially reported to be employed as the first step in the isolation of cell contents, but only recently a number of studies have been focusing on enhancing microbial productivity with ultrasonic irradiation (Mason, 1990). Wood *et al.* (1997) found ultrasonic irradiation caused disintegration of cellulose fibers length, thus increasing the surface area available for enzymatic attack. Aliyu and Hepher (2000) also showed a positive effect of aeration and sonoenergy on cellulose degradation but indicated that variation in temperature, pH and also cross-contamination from other microorganisms would influence hydrolysis performance. Increasing the irradiation intensity could markedly enhanced the hydrolysis rate for most substrates (Imai *et al.*, 2004; Li *et al.*, 2004), with the exception of newspaper. The reason for the adverse effect may be due to impurities present in newspaper.

So far, only bench scales studies are known to have been conducted. More positive results have to be produced before ultrasound can be considered promising enough to be used in a production-scale system for the saccharification of lignocellulosic materials.

2.4.2 Chemical Pretreatments

2.4.2.1 Acids

Acids initially served as catalysts for hydrolysis of cellulose, rather than as reagents for pretreatment, as mentioned previously in **Section 2.2.1**. These days, acid pretreatment techniques are used prior to enzymatic hydrolysis.

Among all the acids, H_2SO_4 has been the most extensively studied, apparently because it is effective. Torget *et al.* (1990) used a batch dilute H_2SO_4 pretreatment process on short rotation woody and herbaceous crops and showed that significantly high xylose yields can be obtained, approaching 80 % of theoretical. Meanwhile, Thompson *et al.* (1991) also pretreated mixed hardwood with dilute H_2SO_4. He observed that the crystallinity index increased as a result of the pretreatment process. This indicated that the amorphous cellulose fractions have been hydrolysed, leaving the recalcitrance crystalline fraction behind. By using dilute H_2SO_4 at different concentrations, i.e. 0.06 % and 0.5 %, Grohman *et al.* (1995) determined that orange peel solids favoured pretreatment with higher acid concentration, but at the same time the economic aspect must be considered.

H_3PO_4 has also been successfully developed for pretreatment of lignocellulosic materials. Wei *et al.* (1996) reported that the extent and the rate of hydrolysis are proportional to acid concentration. At H_3PO_4 concentration of 81 % or less, cellulose undergoes an interfibrillar swelling, whereas at a concentration of 90 %, the crystallinity of cellulose is affected. Alternatively, Nikolov *et al.* (2000) investigated the possibility of using low concentration of 0.25 % H_3PO_4 on delignified waste-cellulose fibers. Compared to the control, the results obtained after pretreatment showed that cellulose was extensively hydrolysed by the cellulase (degree of degradation was 80-85 %).

Although the acid pretreatment process is relatively cheap, has a lower degree of toxicity (compared to other inorganic mineral acids) and can significantly improve hydrolysis process, it requires corrosion-resistant construction materials for reactors and gypsum materials. Moreover, pH neutralisation is required for further downstream processes and fermentation.

2.4.2.2 Alkalis

Numerous studies on alkaline pretreatment have been applied to enhance lignocellulosics digestibility and delignification process. Dilute NaOH treatment of lignocellulosics causes swelling, followed by an increase in accessible surface area, decrease in crystallinity, separation of linkages between lignin and cellulose and disruption of the lignin structure (Fan *et al.*, 1987). Koullas *et al.* (1992) used NaOH to pretreat corn husks and concluded that 60.6 % of lignin and 71.6 % of hemicellulose was solubilised and the crystallinity index was increased. Alkaline peroxide pretreatment conducted on mixed hardwood (90 % birch and 10 % maple) showed a moderate removal of lignin and hemicellulose as well as a large increase in the swelling and surface area of cellulose (Thompson *et al.*, 1991). Ammonia pretreatment can also be used to remove lignin. An aqueous ammonia recycled percolation process was employed for pretreatment of corn cobs or stover mixture and switch grass and it was discovered that the delignification efficiency were 60-80 % and 65-85 % respectively (Sun and Cheng, 2002).

The effectiveness of alkaline pretreatment seems to vary, depending on factors such as substrate composition and treatment condition. Generally, alkaline pretreatment is more effective on agricultural residues and herbaceous crops rather than on wood materials. In comparison with acid-based pretreatment, the cost of alkaline-based pretreatment may be higher and the concentration of alkali used is pretty much the same or higher than that of acid.

2.4.2.3 Solvent

In organosolv processes, the use of an organic or inorganic solvent such as ethanol, methanol, and acid or alkali catalyst is mainly for delignification and solubilisation processes. When a catalyst is used, hemicellulose solubilisation improves and the enzymatic hydrolysis of pretreated lignocellulosics is enhanced (Chum *et al.*, 1988). Sun and Cheng (2002) pointed out that addition of catalyst above 185 °C was found to be less effective for the delignification process. Papatheofanous *et al.* (1996) extracted lignin from wheat straw with a two-stage acid-catalysed process. Their results have indicated that 50 % of straw hemicellulose was hydrolysed by H_2SO_4 (first stage), followed by delignification by ethanol (second stage). This led to high lignin removal of more than 70 %. With this two-stage process, high overall saccharification yield and good pulp mechanical properties were achieved. However, solvents used in the process have to be removed from the system as they may be inhibitory to microbial growth, substrate digestibility and fermentation. Therefore, organosolv processes for pulping are far too costly to replace the conventional pulping method.

2.4.3 Biological Pretreatments

In biological pretreatment processes, brown rots, white rots and red rots are utilised to degrade lignin in lignocellulosic materials. Brown rots mainly attack cellulose, whereas white rots and red rots attack both lignin and cellulose. Akin *et al.* (1995) showed that biodegradation of Bermuda grass by white-rot fungi using *Ceriporiopsis subvermispor*a and *Cyathus stercoreus* after six weeks were improved by 29-32 % and 63-77 % respectively. Hatakka (1983) found that 35 % of wheat straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks. Similar results were obtained by *Pycnoporus cinnabarinus* 115 and *Phanerochaete sordida* 37 in four weeks. In another study, losses of lignin and cellulose in wheat straw due to the action of *Polyporus adustus* were reported to be 22 % and 14 % respectively after 30 days and 40 % and 32 % respectively after 70 days (Fan *et al.*, 1987). In order to prevent the loss of cellulose, a cellulase-less mutant of white-rot fungi was developed for specific lignin degradation. Other lignin-solubilising microorganisms such as lacasses and polyphenol oxidases can also render lignocellulosics amenable to cellulase digestion.

Biological pretreatment has the advantages of working under environmentally friendly conditions and low energy input. However, relatively slow conversion has prevented the use of biological pretreatments in large scale industrial processes.

2.5 Surfactant Addition

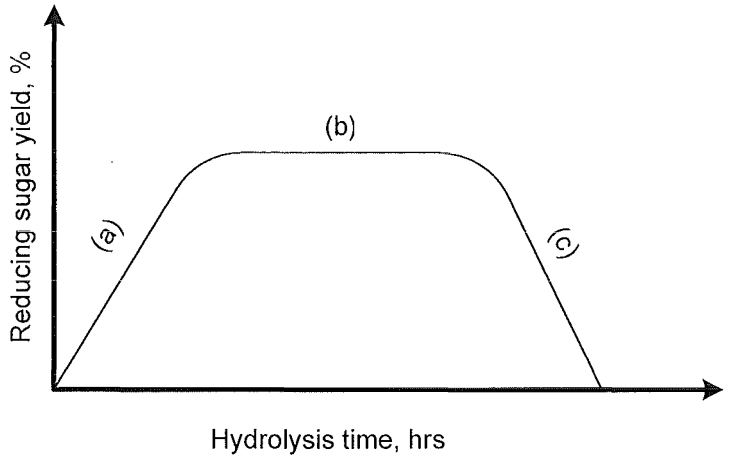


Figure 2.4 Typical digestion curve of enzymatic hydrolysis of cellulose

Figure 2.4 shows that enzymatic hydrolysis of cellulose can be characterised as a 'batch' trend, where (a) a high rate of rapid release of reducing sugars is initially observed, (b) a saturation point is reached and this is followed by (c) a declining rate of sugar production as the reaction proceeds. Relatively high cellulase loadings are required to enhance the hydrolysis rate, but would significantly increase the overall process cost. Thus, a means of increasing cellulase efficiency is imperative for reduction of cellulase consumption.

Enhancement of cellulose hydrolysis by adding surfactants has been reported by several authors (Kaar and Holtzapple, 1998; Kurakake *et al.*, 1994). Wu and Ju (1998) worked on dilute-acid pretreated waste newsprint using 2 % of the surfactant Pluronic F68 with 2 g/l cellulase. The cellulose conversion reached 52 %, compared to 48 % conversion with 10 g/l cellulase in a surfactant-free system. Positive effects of surfactant addition have also been observed using different lignocellulosic materials such as bagasse (Kurakake *et al.*, 1994), steam-pretreated spruce (Eriksson *et al.*, 2002) and tissue paper, which is made of Avicel (Ooshima *et al.*, 1986). It has been reported that non-ionic surfactants increase cellulose hydrolysis, whereas charged surfactants decrease the hydrolysis rate (Eriksson *et al.*, 2002).

Different mechanisms for the positive effect of surfactant addition to the enzymatic hydrolysis of cellulose have been proposed. According to Helle *et al.* (1993), the surfactant could modify the substrate structure, thereby allowing cellulase to attack the cellulose surface. Kaar and Holtzapple (1998) found that surfactant acts as an effector that aids the interaction of the

substrate and the cellulase by either making the substrate more suitable for cellulase attachment or facilitating the release of cellulase back to the aqueous environment once the reaction has occurred. Others reported that the surfactant could also increase the stability of the cellulase by reducing thermal denaturation during hydrolysis (Kaar and Holtzapple, 1998; Kim *et al.*, 1982). Recently, it has been proposed by Eriksson *et al.* (2002) that surfactant adsorption onto lignin prevents unproductive binding of cellulase onto lignin. The study showed that with the addition of the non-ionic surfactant Tween 20, it was possible to lower the cellulase loading by 50 % while at the same time, retaining the hydrolysis yield.

Although non-ionic surfactants are known as valuable additives for enhancing cellulose hydrolysis, the recommended pretreatment conditions remain unchanged regardless of whether surfactants were added during the hydrolysis (Kaar and Holtzapple, 1998).

3 MATERIALS & METHODS

The materials, experimental procedures and statistical design method used during the work are described in this chapter.

3.1 Substrate

Office paper (photocopy and printing paper) and newspaper collected within the campus of University of Canterbury, New Zealand were used as substrate. These materials were cut into pieces of 2 cm x 2 cm before use.

3.2 Cellulase

Two different batches of *T.reesei* cellulase complex (EC 3.2.1.4) (*Sigma-Aldrich Co., product number C-8546*) were used without further purification. The bottles had the following specified hydrolytic activities: 8.5 units/mg solid and 8.1 units/mg solid respectively. According to a *Sigma-Aldrich Catalogue (2002-2003)*, one unit is equal to the release of 1.0 μmole of glucose from cellulose in an hour at pH 5.0 at 37 °C (2 hours incubation time).

3.3 Surfactant

Tween 80, chemically known as *polyoxyethylenesorbitan monoleate*, (*Sigma-Aldrich Co., product number P-8074*) was prepared into solutions of different concentrations. Surfactant stock solution in citrate buffer was prepared at 0, 2, 4, 6 and 8 g/L for office paper and 0, 3, 6, 9, and 12 g/L for newspaper in the incubation mixtures.

3.4 Incubator

The incubator used was a *model OM11* unit from *Ratek Instruments Co.* It had a temperature range of 7 - 75 °C and a mixing control range of 40 - 400 rpm. The incubator had a 400 mm x 400 mm platform equipped with bar racks that could be moved horizontally. Conical flasks were slotted in between rubber-covered stainless steel bars racks secured on the platform.

3.5 pH Measurement

pH analyses were determined using a *Hanna HI131* pH electrode connected to a *Hanna pH211* electronics readout device. To compensate for temperature, the meter was calibrated at the given temperature of the sample using buffer solutions of pH 4.01 ± 0.01 and pH 7.01 ± 0.01.

3.6 Spectrophotometer

The spectrophotometer used was a UV-visible photodiode array spectrophotometer, *model MultiSpec-1501* from *Shimadzu Co.* The unit could cover wavelengths ranging from 190 to 800 nm, depending on the color intensity of a reacted sample. It was able to measure the entire UV and visible spectra almost instantaneously. At the beginning of every session, the spectrophotometer had to be calibrated against a 'zero' value by measuring the absorbance of a 'blank' sample before transferring the actual sample into the cell. Data was then transferred to the bundled Windows-based operation software.

3.7 Dinitrosalicylic Acid (DNS) Reagent Solution

300 g of sodium potassium tartrate and 8 g of sodium metabisulfite were dissolved with approximately 500 mL of deionised water in a 1 L beaker. Sodium metabisulfite was added to absorb dissolved oxygen that may interfere with glucose oxidation over time. Another solution was prepared using 16 g of sodium hydroxide in about 200 mL of deionised water, followed by 10 g of 3,5-dinitrosalicylic acid. The dissolution for this second solution was aided by heat and constant stirring until it was complete.

The two solutions were then combined and filled with deionised water up to 1 L. The beaker was covered with a piece of aluminum foil to prevent loss of the DNS reagent due to evaporation and stored at 2-8 ^{o}C prior to use. A list of reagents required to prepare the DNS reagent is shown in **Table 3.1**.

Table 3.1 List of reagents used to prepare the DNS regent solution

Reagents	Product no.	Amount	Manufacturer
Sodium potassium tartrate	S-6170	300 g	Sigma-Aldrich Co.
Sodium metabisulfite	S-1516	8 g	Sigma-Aldrich Co.
Sodium hydroxide	-	16 g	-
3,5-dinitrosalicylic acid	C-0550	10 g	Sigma-Aldrich Co.
Deionised water	-	1 L	-

3.8 Citrate Buffer Solution

A 1 M citrate buffer stock solution was prepared by adding 105 g of citric acid monohydrate and 0.025 g of sodium azide to 375 mL of deionised water in a 500 mL volumetric flask. Sodium azide was added to prevent microbial growth during incubation. The initial solution pH was 1.9. The solution was then topped up to 500 mL with deionised water. 0.1 M sodium hydroxide was added to the solution until it reached a pH of 4.5. 0.5 mM citrate buffer stock solution was prepared by diluting 1 M citrate buffer stock solution with deionised water in a 500 mL volumetric flask. The pH was adjusted to 4.8, using either 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The 1 M and 0.1 M citrate buffer stock solutions were kept at 2-8 ^{o}C before use. Reagents needed to prepare citrate buffer solution are listed in **Table 3.2**.

Table 3.2 List of reagents used to prepare the citrate buffer solution

Reagents	Product no.	Amount	Manufacturer
Citric acid monohydrate	C-1909	105 g	Sigma-Aldrich Co.
Sodium azide	S-2002	0.025 g	Sigma-Aldrich Co.
0.1 M Hydrochloric acid	-	A few drops	-
0.1 M Sodium hydroxide	-	A few drops	-
Add deionised water to	-	1 L	-

3.9 Glucose Standards

A 2 g/L glucose standard solution was prepared by dissolving 0.5 g glucose in 250 mL of 50 mM citrate buffer solution. From this standard solution, glucose solutions having concentrations of 0.0, 0.4, 1.0, 1.2, 1.4, and 1.6 g/L were prepared by further dilutions with the citrate buffer. A 'blank' sample or 'zero' absorbance was also prepared using the citrate buffer only.

3.10 Standard Curve

The glucose standards readings were determined using the spectrophotometer. Once the data was obtained, a standard curve for the DNS analysis was plotted in a spreadsheet (refer to **Appendix A**) based on the standard glucose solutions and blank sample (0.0, 0.4, 1.0, 1.2, 1.4 1.6 and 2.0 g/L) versus the plotted absorbance (in nm). The standard curve was found to be linear.

3.11 Pretreatment of Paper Substrate

The phosphoric acid used for pretreatment was in crystal form (*Sigma-Aldrich Co. product number 310271*). The acid was prepared in several concentrations: 2, 4, 6 and 8 g/L. For example, a phosphoric acid solution of 2 g/L was prepared by dissolving 2 g of phosphoric acid with 1 L of deionised water in a 1 L beaker.

Paper, in pieces each measuring 2 cm x 2 cm was weighed by an electronic balance accurate to 0.0001 g. The weight of paper needed was different for pretreatment at the different phosphoric acid concentrations. For each concentration of the phosphoric acid, a predetermined amount of papers were slowly added into the acid while the solution was gently agitated with a magnetic stirrer. The pretreatment took place for an hour at room temperature (21 ± 1 ^{o}C).

After the pretreatment, the phosphoric acid solution was poured off and the beaker (with the paper inside) was filled with deionised water. The contents were then gently agitated for 10 minutes before the pH was measured. If pH was lower than neutral pH, the water was poured off and the beaker was refilled with deionised water, and again the contents would be agitated for another five minutes. This was repeated until the pH was neutral.

The wet paper pieces were then oven-dried at 70 °C for 10 minutes. The dried paper pieces were then cut into smaller 0.2 cm x 0.2 cm size pieces (see **Figure 3.1**) and kept in separately labelled (based on different concentration and substrate types) screw-cap bottles.

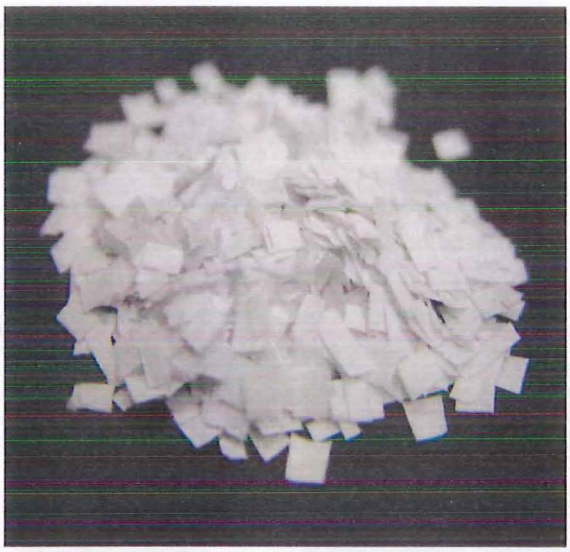


Figure 3.1 Newspaper in equal sizes of 2 X 2 mm square

3.12 Experimental Procedures

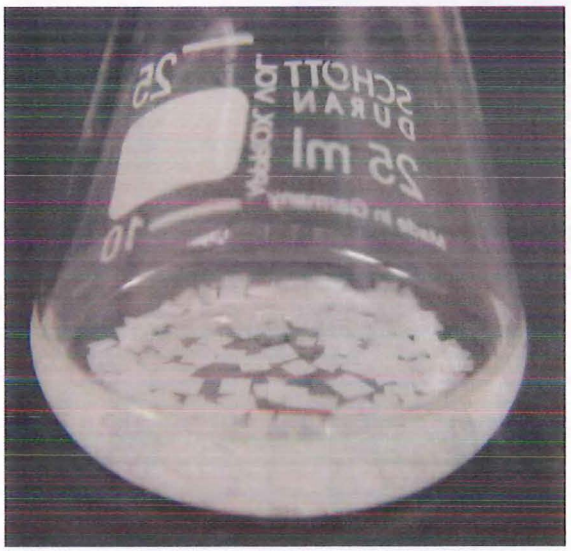
Enzymatic hydrolysis was carried out using 50 mg of substrate in 0.5 mM citrate buffer (pH 4.8) in 25 mL conical flasks. The hydrolysis experiments were first carried out by transferring the pretreated substrates into the separately labelled conical flasks, followed by addition of the Tween 80 solution. The flasks were incubated at 50°C and 110 rpm for 10 minutes before the cellulase complex from *T.reesei* was added (see **Figure 3.2 (a)**). Each flask was covered with aluminium foil to prevent loss of liquid due to evaporation. The experiments were run for 20 hours. The liquid samples were withdrawn periodically (see **Figure 3.2 (b)**) at intervals of 4, 8, 12, 16 and 20 hours and transferred into appropriately labelled 7 mL screw-cap tubes (see **Figure 3.2 (c)**).

The hydrolysis reaction was terminated by leaving the reaction samples in a heated water bath at 90 °C for 10 minutes to inactivate the enzyme. The samples were then allowed to cool to room temperature (see **Figure 3.2(d)**). 1.6 mL of the reaction mixtures were transferred into 2 mL micro centrifuge tubes and centrifuged at 10,000 rpm for 5 minutes. Each 1 mL of supernatant obtained was pipetted and transferred into separately labelled 25 mL screw-cap bottles. 1 mL of

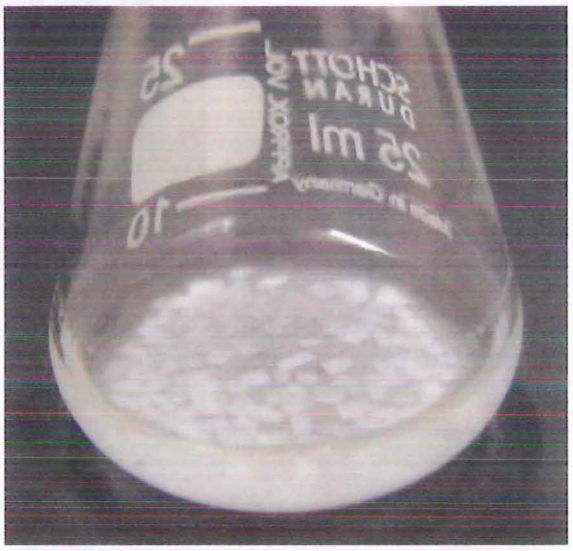
DNS reagent was then added to each bottle. For the buffered sample or blank, 1 mL of citrate buffer was mixed with 1.0 mL DNS reagent instead (see **Figure 3.2(e)**). Each bottle was mixed rapidly using a vortex mixer.

The samples were then heated in the boiling water bath for 10 minutes. A reddish-brown color would develop for samples containing glucose. The samples were cooled at room temperature and 10 mL of deionised water was added prior to analysis of total reducing sugars (see **Figure 3.2(f)**).

(a)

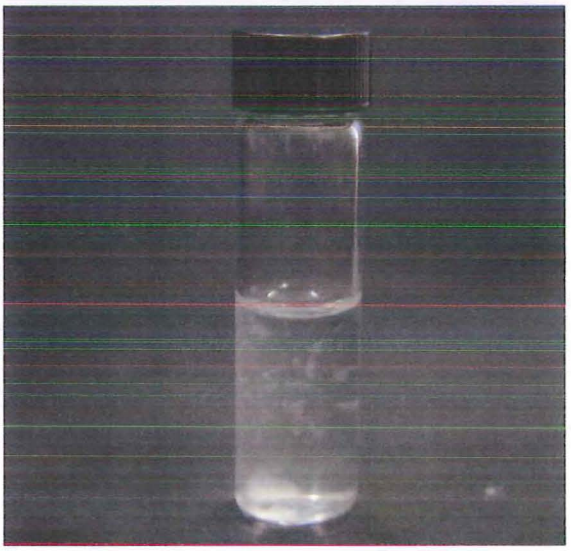


(b)



(c)

(d)



(e)

(f)

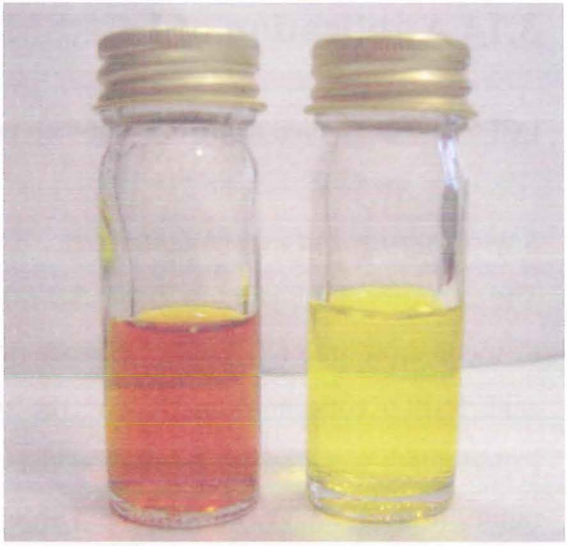


Figure 3.2 (a) Sample before incubation; (b) Sample after 8 hours incubation; (c) Withdrawn samples in 7 mL screw-cap tubes; (d) Sample after being heated for 10 minutes; (e) 1.0 mL DNS reagent + 1.0 mL sample (left) and 1.0 mL DNS reagent + 1.0 mL citrate buffer (right) before boiling; (f) After boiling, a reddish-brown colour developed in samples containing sugar. 10 mL deionised water was then added to each screw-cap bottle

3.13 Analytical Techniques

The total reducing sugars were estimated using the dinitrosalicylic acid (DNS) method (Miller, 1959). The buffered sample (blank) was filled into a 10 mm quartz cuvette and placed into the cell holder. The UV-VIS spectrophotometer was zeroed at 546 nm. The intensity of the developed color was measured almost instantaneously. The buffered sample was later removed from the cell holder and the cell was replaced with the reacted sample. Using the absorbance reading recorded by the spectrophotometer, sugar concentration was calculated from the calibration curve made earlier.

3.14 Application of LCRSM

LCRSM was applied using the four-step process as described by Allen and Yu (2002): -

Step 1 (Set-up and experimentation)

The experimental factors in **Table 3.3 (a)** or **(b)** and **Table 3.4** were chosen to study the effects of incubation time (X_1), enzyme/paper ratio (X_2), surfactant concentration (X_3) and phosphoric acid, H_3PO_4 concentration (X_4) on the sugar yield (Y) in the enzymatic hydrolysis step. The experiments were set up by scaling the experimental design presented in **Table 3.5** using the ranges in **Table 3.3 (a)** or **(b)** and **Table 3.4**. The scaled inputs are shown in **Table 3.6** and the tests were performed randomly to avoid biased results. There were 14 runs in the array (see **Table 3.6 (a)** and **(b)**), including three repeated runs to evaluate the variation of the data in the modelling and analysis procedure.

Table 3.3 Response, coded and actual four factor, five levels used in enzymatic hydrolysis of (a) office paper and (b) newspaper

(a)

Y = Response (%)	Sugar yield				
Levels	-1.0	-0.5	0.0	0.5	1.0
Factors					
X_1 = Reaction time (h)	4.0	8.0	12.0	16.0	20.0
X_2 = Enzyme/paper ratio (%)	2.0	6.0	10.0	14.0	18.0
X_3 = Surfactant concentration (g/L)	0.0	2.0	4.0	6.0	8.0
X_4 = H_3PO_4 concentration (g/L)	0.0	2.0	4.0	6.0	8.0

(b)

Y = Response (%)	Sugar yield				
Levels	-1.0	-0.5	0.0	0.5	1.0
Factors					
X_1 = Reaction time (h)	4.0	8.0	12.0	16.0	20.0
X_2 = Enzyme/paper ratio (%)	2.0	6.0	10.0	14.0	18.0
X_3 = Surfactant concentration (g/L)	0.0	3.0	6.0	9.0	12.0
X_4 = H_3PO_4 concentration (g/L)	0.0	2.0	4.0	6.0	8.0

Table 3.4 Amount of cellulase required at the five levels for office paper and newspaper

Levels	-1.0	-0.5	0.0	0.5	1.0
Enzyme/paper ratio (%)	2.0	6.0	10.0	14.0	18.0
Amount of enzyme (mg)	1.0	3.0	5.0	7.0	9.0
Amount of paper (mg)	50.0	50.0	50.0	50.0	50.0

Table 3.5 LCRSM for office paper and newspaper (a) The start-up design in coded (-1.0, 1.0) levels, (b) the model forms and (c) the follow-up runs that were needed to achieve accuracy goals

(a)

Run	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
1	-0.5	-1.0	-0.5	1.0
2	1.0	1.0	-1.0	1.0
3	-1.0	1.0	1.0	1.0
4	1.0	-1.0	-0.5	-0.5
5	0.0	0.0	-1.0	0.0
6	0.0	1.0	0.0	0.0
7	-0.5	-1.0	1.0	-0.5
8	-1.0	0.0	0.0	0.0
9	1.0	1.0	1.0	-1.0
10	-1.0	1.0	-1.0	-1.0
11	0.0	0.0	0.0	-1.0
12	0.5	-0.5	0.5	0.5
13	0.5	-0.5	0.5	0.5
14	0.5	-0.5	0.5	0.5

(b)

Model form #1: $\beta_o + \beta_{X1} X_1 + \beta_{X2} X_2 + \beta_{X3} X_3 + \beta_{X4} X_4 + \beta_{X1\,2} X_1^2 + \beta_{X2\,2} X_2^2 + \beta_{X3\,2} X_3^2 + \beta_{X1\,X2} X_1 X_2 + \beta_{X1\,X3} X_1 X_3 + \beta_{X2\,X3} X_2 X_3$

Model form #2: $\beta_o + \beta_{X1} X_1 + \beta_{X2} X_2 + \beta_{X3} X_3 + \beta_{X4} X_4 + \beta_{X1\,2} X_1^2 + \beta_{X2\,2} X_2^2 + \beta_{X4\,2} X_4^2 + \beta_{X1\,X2} X_1 X_2 + \beta_{X1\,X4} X_1 X_4 + \beta_{X2\,X4} X_2 X_4$

Model form #3: $\beta_o + \beta_{X1} X_1 + \beta_{X2} X_2 + \beta_{X3} X_3 + \beta_{X4} X_4 + \beta_{X1\,2} X_1^2 + \beta_{X3\,2} X_3^2 + \beta_{X4\,2} X_4^2 + \beta_{X1\,X3} X_1 X_3 + \beta_{X1\,X4} X_1 X_4 + \beta_{X3\,X4} X_3 X_4$

Model form #4: $\beta_o + \beta_{X1} X_1 + \beta_{X2} X_2 + \beta_{X3} X_3 + \beta_{X4} X_4 + \beta_{X2}\,2\, X_2^2 + \beta_{X3}\,2\, X_3^2 + \beta_{X4}\,2\, X_4^2 + \beta_{X2\,X3} X_2 X_3 + \beta_{X2\,X4} X_2 X_4 + \beta_{X3\,X4} X_3 X_4$

(c)

Run	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
15	-1.0	-1.0	-1.0	1.0
16	-1.0	-1.0	-1.0	-1.0
17	-1.0	1.0	1.0	-1.0
18	1.0	1.0	-1.0	-1.0

Table 3.6 The scaled inputs of the four independent variables that influenced the sugar yield and the actual run order of (a, b) office paper and (c, d) newspaper

(a)

Run order	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
2	20.0	18.0	0.0	8.0
9	20.0	18.0	8.0	0.0
5	12.0	10.0	0.0	4.0
14	16.0	6.0	6.0	6.0
8	4.0	10.0	4.0	4.0
1	8.0	2.0	2.0	8.0
11	12.0	10.0	4.0	0.0
10	4.0	18.0	0.0	0.0
3	4.0	18.0	8.0	8.0
7	8.0	2.0	8.0	2.0
12	16.0	6.0	6.0	6.0
4	20.0	2.0	2.0	2.0
6	12.0	18.0	4.0	4.0
13	16.0	6.0	6.0	6.0

(b)

Run	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
15	4.0	18.0	0.0	8.0
16	4.0	2.0	0.0	0.0
17	4.0	18.0	8.0	0.0
18	20.0	18.0	0.0	0.0

(c)

Run order	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
2	20.0	18.0	0.0	8.0
9	20.0	18.0	12.0	0.0
1	8.0	2.0	3.0	8.0
11	12.0	10.0	6.0	0.0
12	16.0	6.0	9.0	6.0
10	4.0	18.0	0.0	0.0
5	12.0	10.0	0.0	4.0
4	20.0	2.0	3.0	2.0
14	16.0	6.0	9.0	6.0
7	8.0	2.0	12.0	2.0
3	4.0	18.0	12.0	8.0
8	4.0	10.0	6.0	4.0
6	12.0	18.0	6.0	4.0
13	16.0	6.0	9.0	6.0

(d)

Run	Reaction time (X_1)	Enzyme/paper ratio (X_2)	Surfactant (X_3)	H_3PO_4 (X_4)
15	4.0	18.0	0.0	8.0
16	4.0	2.0	0.0	0.0
17	4.0	18.0	12.0	0.0
18	20.0	18.0	0.0	0.0

Step 2 (Model selection)

The regression models of each response were produced by fitting the appropriate set of model forms shown in **Table 3.5 (b)**. The fitted model form with the lowest sum of squares error (SSE) or highest R^2 was chosen.

Step 3 (The least-squares coefficient based diagnostic)

In order to determine whether the additional four runs were required, the formula represented by **Equation 3.1** was calculated.

$$\beta_{q,\,est} = \left(\sum_{i}^{q} \beta^2_{i,est} \right)^{1/2} (q-1)^{-1/2} \qquad \textbf{(3.1)}$$

$\beta_{q,\,est}$ is the least-squares estimates of the q = 6 second-order coefficients in the model chosen in Step 2. These included coefficient terms like A^2 and CD, but not first-order terms such as A and B. If the maximum acceptable standard error of prediction or 'plus or minus' goal, $\sigma_{prediction} \leq$ 1.0, 14 runs would have been adequate. Otherwise, it was essential to conduct an additional four runs as described in Step 4.

Step 4 (Additional runs, if necessary)

Since an additional four runs were needed to accomplish the accuracy goals in Step 3, additional experimental runs were performed as specified in **Table 3.6 (b)** or **(d)**. After the experiments, a full quadratic polynomial regression model was fitted in ordinary response surface methods (RSM). The final prediction model obtained was used to study the effects of the input factors on the response.

4 RESULTS & DISCUSSION

4.1 Office Paper

Step 1 (Set-up and experimentation)

The experiments were performed according to the set-up shown in **Table 4.1**. The 14 runs were conducted in a random order. For each run, the values of sugar yield obtained were recorded.

Table 4.1 Enzymatic hydrolysis conditions, actual and predicted response variables for office paper using LCRSM for the actual 14 run order

Run order	Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Actual sugar yield (%)	Predicted sugar yield (%)
2	20.0	18.0	0.0	8.0	79.8	80.0
9	20.0	18.0	8.0	0.0	63.9	63.9
5	12.0	10.0	0.0	4.0	48.4	54.8
14	16.0	6.0	6.0	6.0	50.2	50.0
8	4.0	10.0	4.0	4.0	32.9	30.2
1	8.0	2.0	2.0	8.0	12.9	12.4
11	12.0	10.0	4.0	0.0	60.1	57.4
10	4.0	18.0	0.0	0.0	36.5	37.0
3	4.0	18.0	8.0	8.0	38.6	41.7
7	8.0	2.0	8.0	2.0	15.0	16.9
12	16.0	6.0	6.0	6.0	50.7	50.0
4	20.0	2.0	2.0	2.0	37.9	37.4
6	12.0	18.0	4.0	4.0	62.9	59.1
13	16.0	6.0	6.0	6.0	50.4	50.0

Step 2 (Model selection)

Fit model form #1 (refer to **Appendix B**) had the highest R^2 value among the four fit model forms that were evaluated. This model was used as a tentative model as represented by **Equation 4.1**.

$$Y = + 56.78 + 17.65A + 19.36B + 0.22C - 0.12D - 4.46A^2 - 11.08B^2 - 6.45C^2 - 0.27AB - 4.35AC - 3.42BC \quad \textbf{(4.1)}$$

where:

Y= Sugar yield (%)

A= Reaction time (h)

B= Enzyme/paper ratio (%)

C= Surfactant concentration (g/L)

D= H_3PO_4 concentration (g/L)

Step 3 (The least-squares coefficient based diagnostic)

To determine whether additional runs were needed, $\beta_{q,\,est}$ was calculated using **Equation 4.2**.

$$\beta_{q,\,est} = \left(\sum_i^q \beta^2_{i,est}\right)^{1/2} (q-1)^{-1/2} \quad \textbf{(4.2)}$$

$$= [(-4.46)^2 + (-11.08)^2 + (-6.45)^2 + (-0.27)^2 + (-4.35)^2 + (-3.42)^2]^{1/2} \times (6-1)^{-1/2}$$

$$= (214.96)^{1/2} \times (5)^{-1/2}$$

$$= 6.56$$

The maximum acceptable standard error of prediction, $\sigma_{prediction}$ was set at 1.0. Since $\beta_{q,\,est} > \sigma_{prediction}$, additional experiments were necessary in order to meet the model accuracy goal.

Step 4 (Additional runs)

As determined in step 3, an additional four runs were conducted, again in random order. For each run, the values of sugar yield obtained were recorded, as shown in **Table 4.2** below.

Table 4.2 Enzymatic hydrolysis conditions, actual and predicted response variables for office paper using LCRSM for the actual 18 run order

Run order	Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Actual sugar yield (%)	Predicted sugar yield (%)
2	20.0	18.0	0.0	8.0	79.8	80.2
9	20.0	18.0	8.0	0.0	63.9	64.4
5	12.0	10.0	0.0	4.0	48.4	48.6
18	20.0	18.0	0.0	0.0	63.7	63.0
8	4.0	10.0	4.0	4.0	32.9	33.2
1	8.0	2.0	2.0	8.0	12.9	13.2
11	12.0	10.0	4.0	0.0	60.1	60.4
10	4.0	18.0	0.0	0.0	36.5	37.3
3	4.0	18.0	8.0	8.0	38.6	39.1
7	8.0	2.0	8.0	2.0	15.0	15.3
12	16.0	6.0	6.0	6.0	50.7	50.1
4	20.0	2.0	2.0	2.0	37.9	38.2
6	12.0	18.0	4.0	4.0	62.9	62.7
13	16.0	6.0	6.0	6.0	50.4	50.1
14	16.0	6.0	6.0	6.0	50.2	50.1
15	4.0	18.0	0.0	8.0	44.7	44.0
16	4.0	2.0	0.0	0.0	10.5	10.0
17	4.0	18.0	8.0	0.0	35.1	34.4

Comparison amongst the four candidates showed that fit model form #2 had the highest R^2 value of 0.9849 (refer to **Appendix B**).

Next, the data was reanalysed with a full quadratic model to determine whether it provided a better model than reduced model form #2. The R^2 value obtained from the full quadratic model was 0.9994 (refer to **Appendix B**), which was an improvement of 0.0145 with the addition of

four additional coefficients. This was achieved without any apparent increase in computational time, suggesting that the full model would be the most appropriate model. Furthermore, **Figure 4.1** shows that the scatter plot of actual against predicted sugar yield for the full model forms a relatively straight pattern, with an R^2 value of 0.9988. In view of this, the full model was selected.

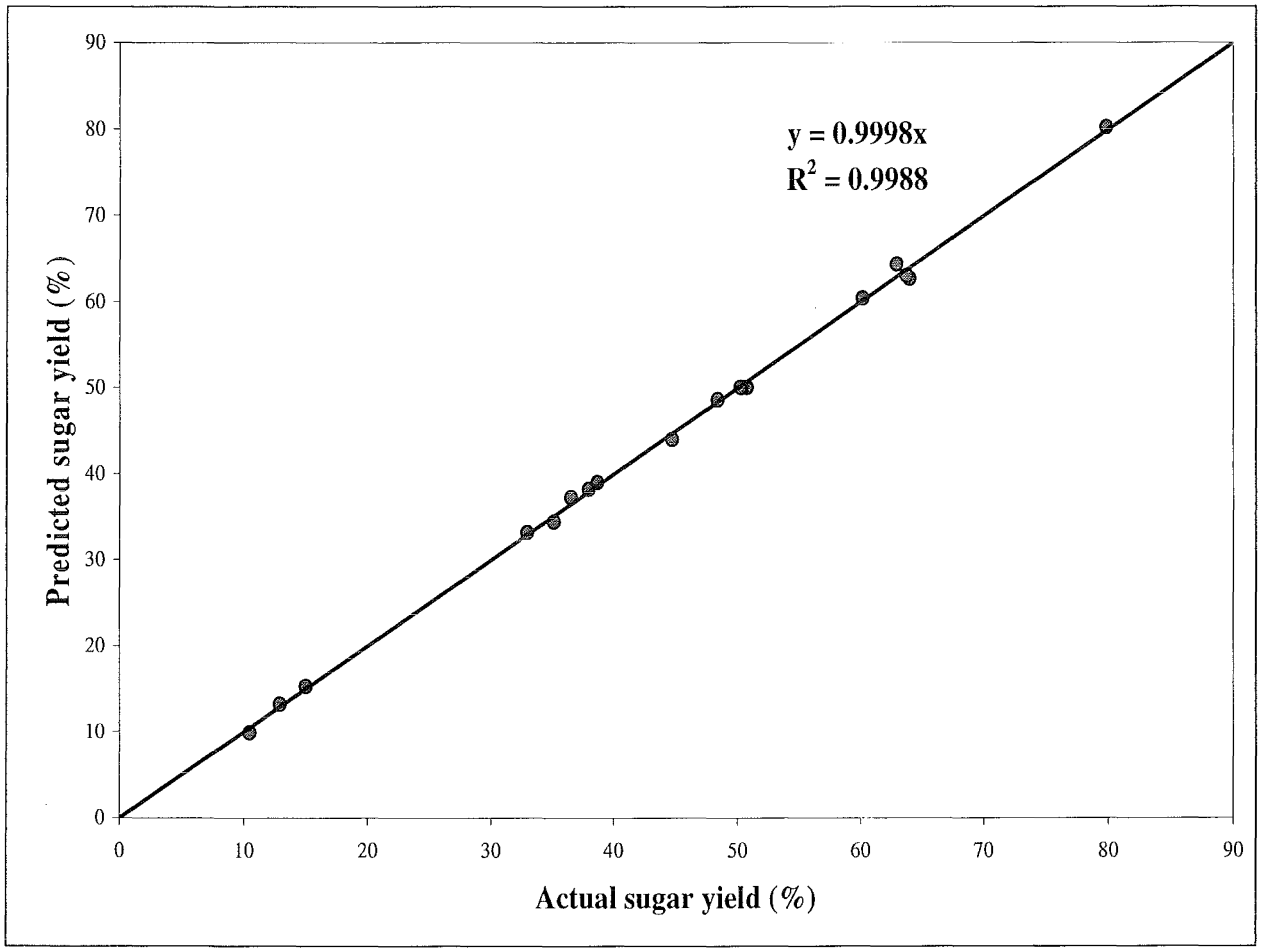


Figure 4.1 Full model plot for actual vs. predicted sugar yield

The final second-order polynomial model in terms of coded factors was obtained as given by **Equation 4.3**.

$$Y = +55.50 + 16.81A + 17.96B + 0.012C - 0.043D - 5.48A^2 - 10.77B^2 - 6.84C^2 + 4.86D^2 - 0.29AB + 1.04AC + 2.61AD - 0.93BC + 5.49BD - 0.53CD \quad (4.3)$$

With the inputs expressed in terms of actual factors, the final prediction model is represented by **Equation 4.4**.

$$\begin{aligned}\text{Sugar yield} = {} & -13.18078 + 3.74549\text{Reaction time} + 5.09555\text{Enzyme/paper ratio} \\ & + 3.45562\text{Surfactant concentration} - 5.00201 H_3PO_4 \text{ concentration} \\ & - 0.085623\text{Reaction time}^2 - 0.16832\text{Enzyme/paper ratio}^2 \\ & - 0.42761\text{Surfactant concentration}^2 + 0.30399 H_3PO_4 \text{ concentration}^2 \\ & - 0.00450395\text{Reaction time Enzyme/paper ratio} \\ & + 0.032609\text{Reaction time Surfactant concentration} \\ & + 0.081437\text{Reaction time } H_3PO_4 \text{ concentration} \\ & - 0.029029\text{Enzyme/paper ratio Surfactant concentration} \\ & + 0.17150\text{Enzyme/paper ratio } H_3PO_4 \text{ concentration} \\ & - 0.033219\text{Surfactant concentration } H_3PO_4 \text{ concentration} \end{aligned} \quad \textbf{(4.4)}$$

A series of experiments were carried out at other conditions within the range of settings listed in **Table 3.3 (a)** and **Table 3.4**. The experimental runs are provided in **Table 4.3.** The resulting regression full model from **Equation 4.4** was analysed by replacing the variable conditions accordingly into the regression equation. The main reason was to observe how closely the full model estimated the actual sugar yield data. The values of the measured response and the predicted response obtained from **Table 4.3** were then plotted, as shown in **Figure 4.2**. The outcome showed that the R^2 value equals 0.9471, which once again verified that the full model was adequate for predicting sugar yield.

Table 4.3 Sugar yield data

Run order	Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Actual sugar yield (%)	Predicted sugar yield (%)
4	20.0	18.0	4.0	4.0	75.2	73.7
6	20.0	10.0	4.0	8.0	69.6	73.6
10	12.0	10.0	4.0	4.0	55.5	54.9
2	20.0	2.0	4.0	4.0	41.1	37.8
9	12.0	10.0	0.0	0.0	50.0	52.4
3	4.0	10.0	0.0	4.0	27.8	26.8
5	4.0	10.0	4.0	8.0	41.0	34.8
1	4.0	18.0	4.0	4.0	46.3	40.1
7	12.0	18.0	4.0	8.0	64.8	72.4
8	4.0	10.0	8.0	4.0	22.7	24.7

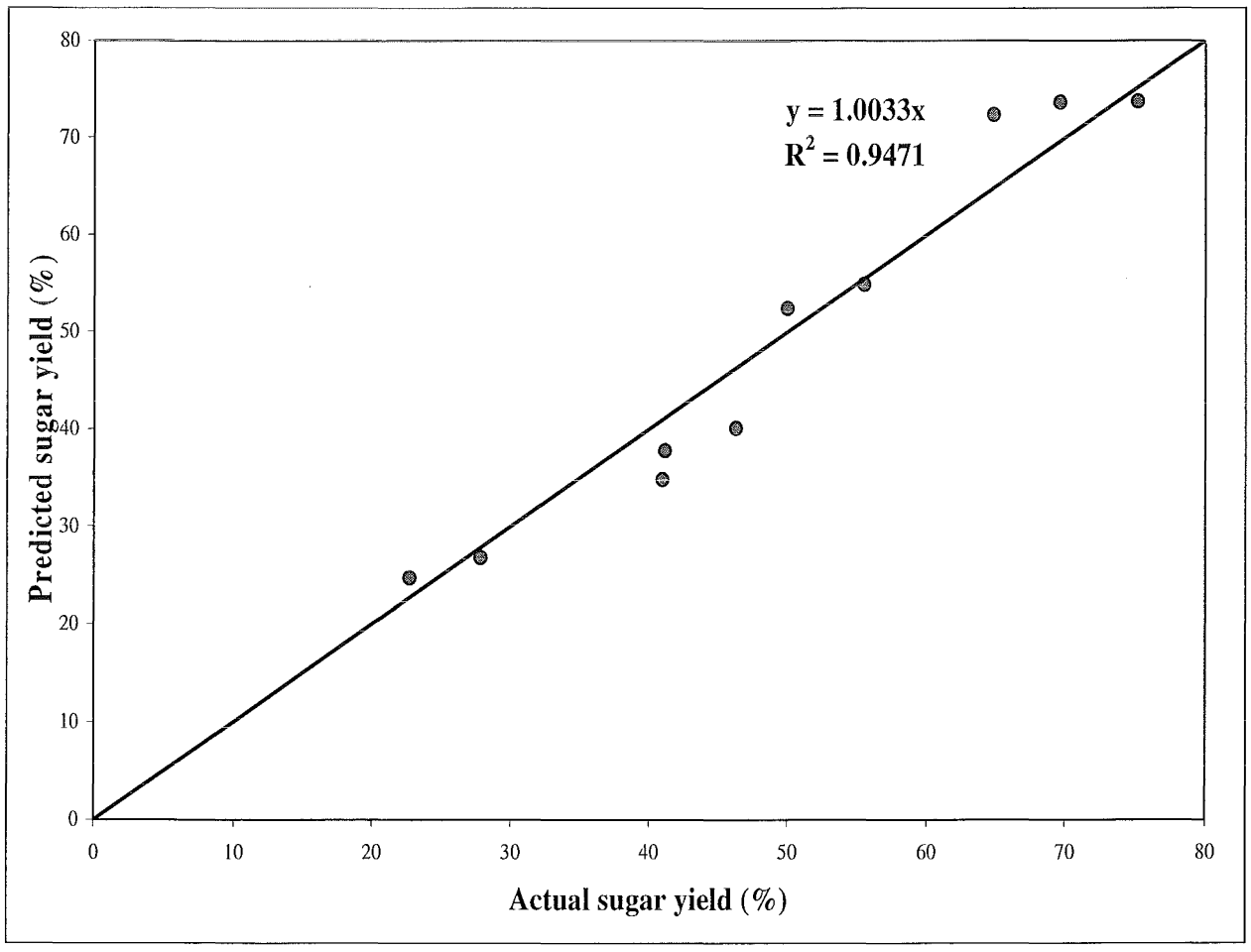


Figure 4.2 Full model plot for actual vs. predicted sugar yield

4.1.1 Scanning Electron Microscope (SEM) Analysis

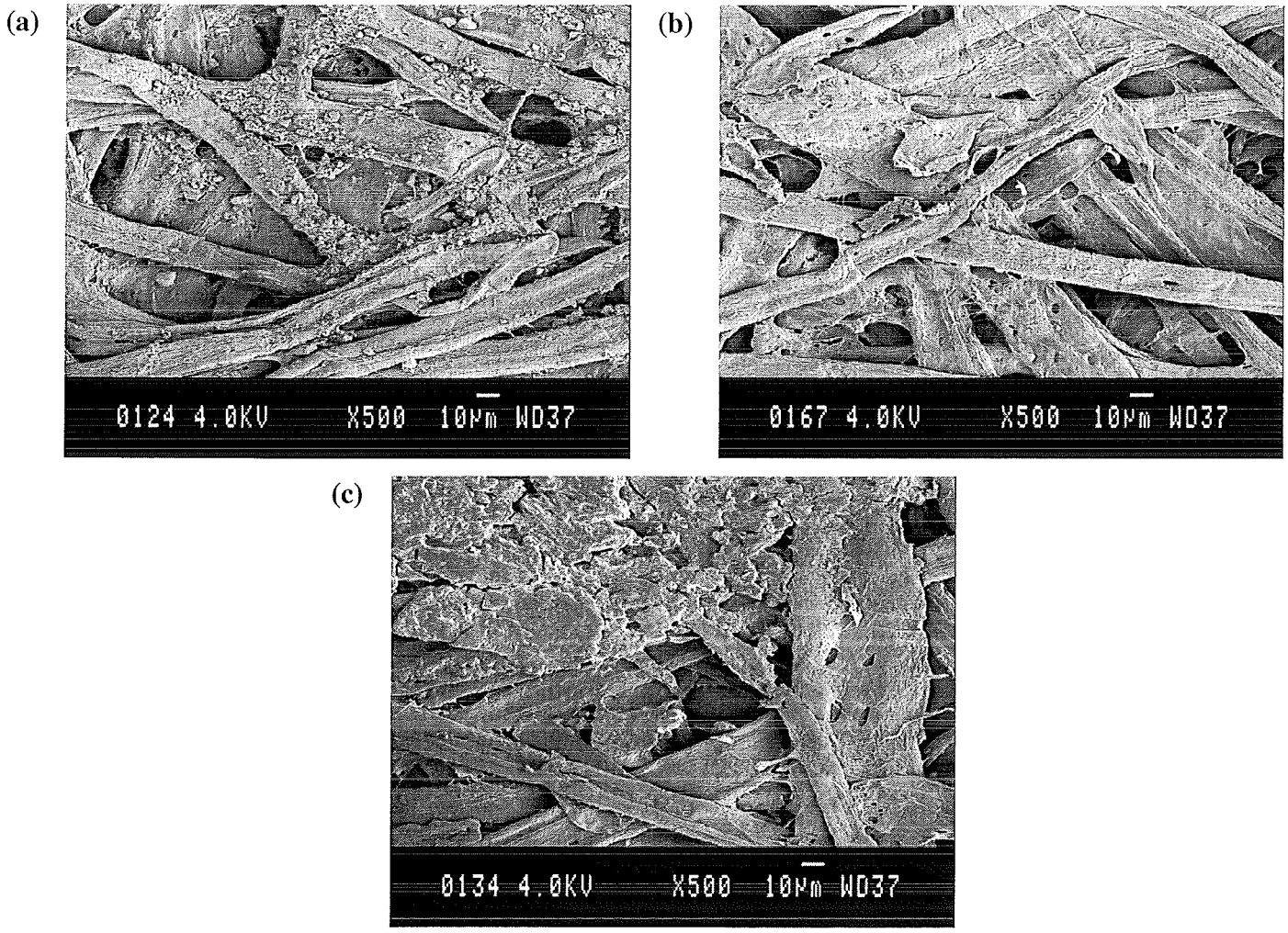


Figure 4.3 SEM-micrographs of unhydrolysed office paper surface with (a) no H_3PO_4 pretreatment (b) 4 g/L H_3PO_4 pretreatment and (c) 8 g/L H_3PO_4 pretreatment

Figure 4.3 shows electron micrographs taken by a TSM-6100 scanning electron microscope of the surface appearances of the office paper with and without pretreatment. It was observed that the control office paper surface consisted of fibers that were very coarse and long. After 4 g/L H_3PO_4 pretreatment was applied, there was a change in the fibers' coarseness. It appeared that the acid had a peeling effect, as particles attached to the fibers' surface were removed; making the surface smoother. Fiber length remained almost the same, but small holes appeared on the surface. With 8 g/L pretreatment, the holes were larger and parts of the fibers that were less coarse were disintegrated into shorter fibers.

Thus, acid pretreatment had the effect of increasing fine fiber content in the substrate, which then influenced the adsorption capacity. It is not, however, the purpose of this chapter to discuss the results based on the geometrical characteristics of fiber structure on office paper.

4.1.2 Response Surface Plots

4.1.2.1 Influence of Four Input Factors on the Overall Sugar Yield

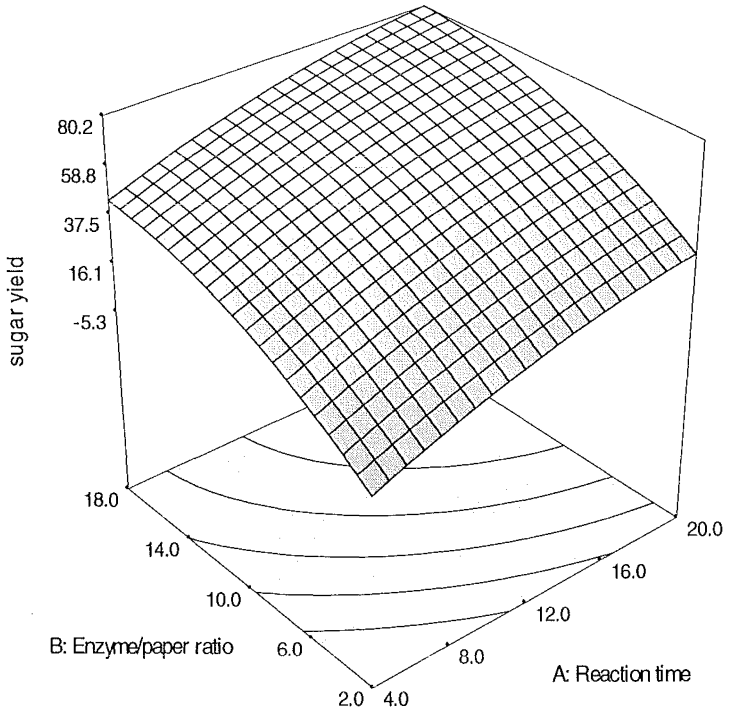


Figure 4.4 Response surface of sugar yield as a function of enzyme/paper ratio and reaction time at surfactant concentration (0 g/L) and H_3PO_4 concentration (8 g/L)

The effects of varying enzyme/paper ratio and reaction time at fixed surfactant concentration (0 g/L) and H_3PO_4 concentration (8 g/L) are shown in **Figure 4.4**. Higher enzyme/paper ratio and longer reaction time had a favourable effect on sugar yield.

Figure 4.5 (a) and **(b)** show that the extent of sugar yield depended on the initial amount of cellulase added. Sugar yield at an enzyme/paper ratio of 2 % was less than sugar yield at an enzyme/paper ratio of 18 %, regardless of the reaction time. Also, it showed that increasing the amount of cellulase would not only increased sugar yield but also resulted in a higher initial hydrolysis rate.

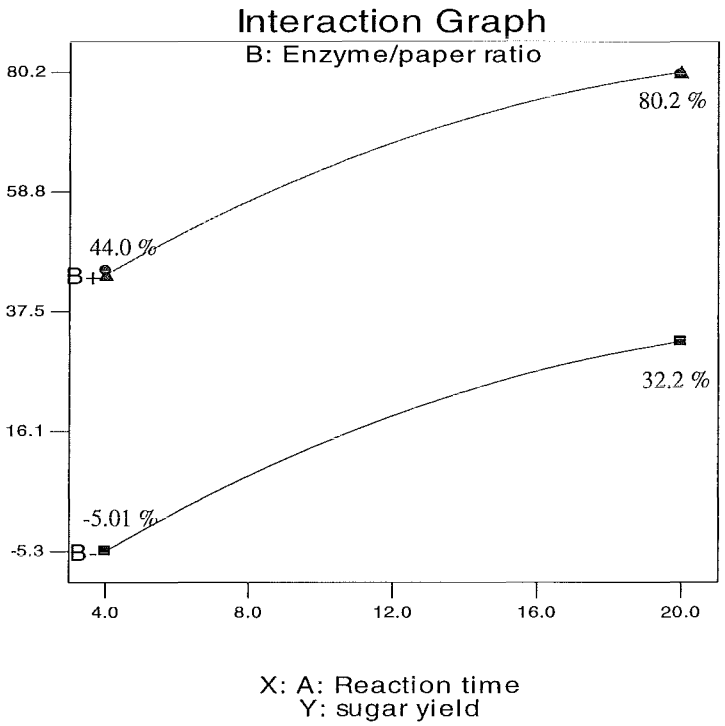
At each enzyme/paper ratio, the initial hydrolysis rate was high, but slowed down as the reaction progressed. This is known as batch hydrolysis, where a rapid release of reducing sugars is followed by a declining pattern as the hydrolysis proceeds. A longer reaction time was required for efficient contact between cellulase and cellulose. This effect became more pronounced as

enzyme/paper ratio and reaction time increased and as a consequence of continuous digestion of cellulose by cellulase. As time continued, cellulase efficiency started to level off despite the increased in total sugar production. This could have been due to the fact that accessible substrate surface area for cellulase to attack had become less due to structural change. Another possibility could be the depletion of substrate in the presence of excess cellulase.

At the lowest enzyme/paper ratio (2 %) and reaction time (4 h) tested, sugar yield was found to be zero (prediction – 5.01 %). This showed that at such a low cellulase concentration and short reaction time, no detectable reaction had occurred.

The optimum amount of enzyme/paper ratio to achieve maximum reducing sugars conversion was at the highest enzyme/paper ratio (18 %) and reaction time (20 h). At this point, the sugar yield was 80.2 %. Further increase of the enzyme/paper ratio or extension of the reaction time would most likely enable more complete hydrolysis to occur.

(a)



(b)

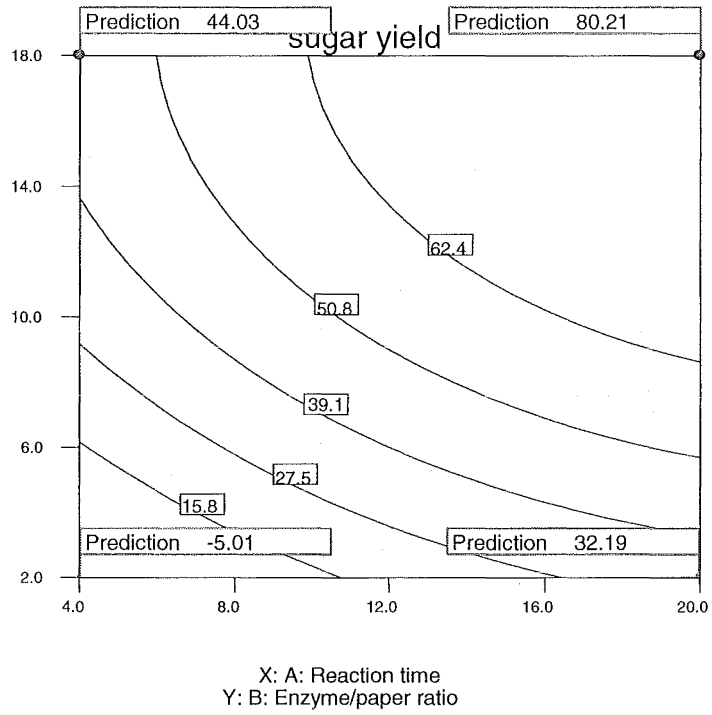


Figure 4.5 (a) Response interaction graph between reaction time and enzyme/paper ratio at (▲) 18 % and (■) 2 % and (b) response contours between the interaction of reaction time and enzyme/paper ratio

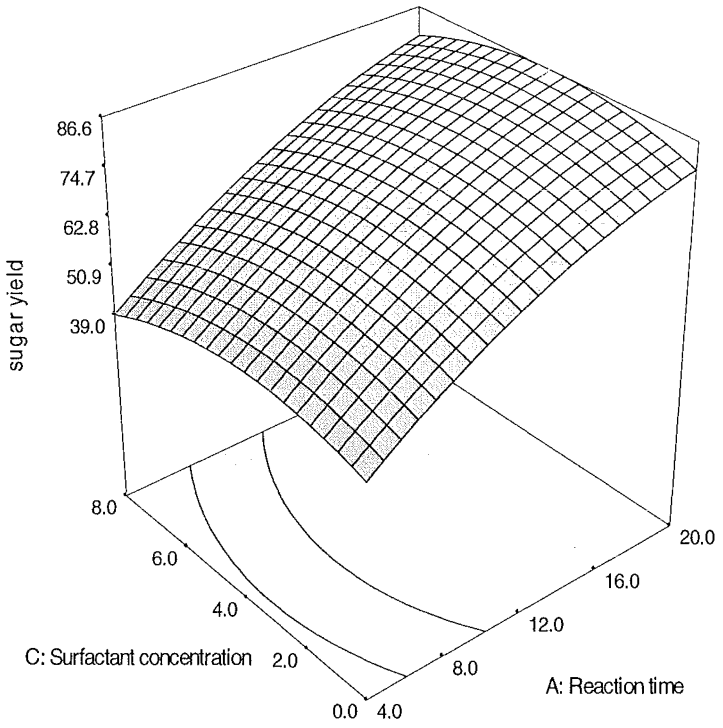


Figure 4.6 Response surface of sugar yield as a function of surfactant concentration and reaction time at enzyme paper/ratio (18 %) and H_3PO_4 concentration (8 g/L)

Figure 4.6 shows the interaction between surfactant concentration and reaction time at fixed enzyme/paper ratio (18 %) and H_3PO_4 concentration (8 g/L). It was apparent that sugar yield changed with increasing reaction time, with only minor influence from surfactant concentration. At a constant reaction time, sugar yield increased from a surfactant concentration of 0 g/L to about 4 g/L, after which the yield deteriorated.

The test result also showed that the highest surfactant concentration tested (8 g/L) gave slightly less sugar yield compared to a surfactant free substrate, as shown in **Figure 4.7 (a)**. For example, at 20 hours reaction time and 8 g/L surfactant concentration, sugar yield was 79.4 % compared to 80.2 % for the condition at 20 hours reaction time and 0 g/L surfactant concentration. The hydrolysis rate for the highest surfactant concentration was also slightly lower compared to a surfactant free substrate.

All the above indicated that surfactant addition had a negligible or adverse effect on the hydrolysis of office paper. Other studies have, however, concluded that the addition of surfactant to the enzymatic hydrolysis is beneficial. All major *T.reesei* adsorbs onto the insoluble substrate through hydrophobic interaction (Palonen *et al.*, 2004) and the extent of saccharification yield depends on its adsorption capability. The hydrophobic residues of the cellulase may bind specifically and non-specifically onto the solid substrate surface. It was suggested that surfactants decreased the unproductive binding of cellulase by occupying the hydrophobic sites of the recalcitrant substrate surface. Such interaction released the non-specifically bound cellulase back to the solution (Eriksson *et al.*, 2002). Kaar and Holtzapple (1998) reported that surfactant addition on corn stover improved cellulose conversion by 42 %. The positive effect of cellulose hydrolysis in the presence of surfactant was also seen on steam-exploded wood (Helle *et al.*, 1993) and newspaper (Castanon and Wilke, 1981).

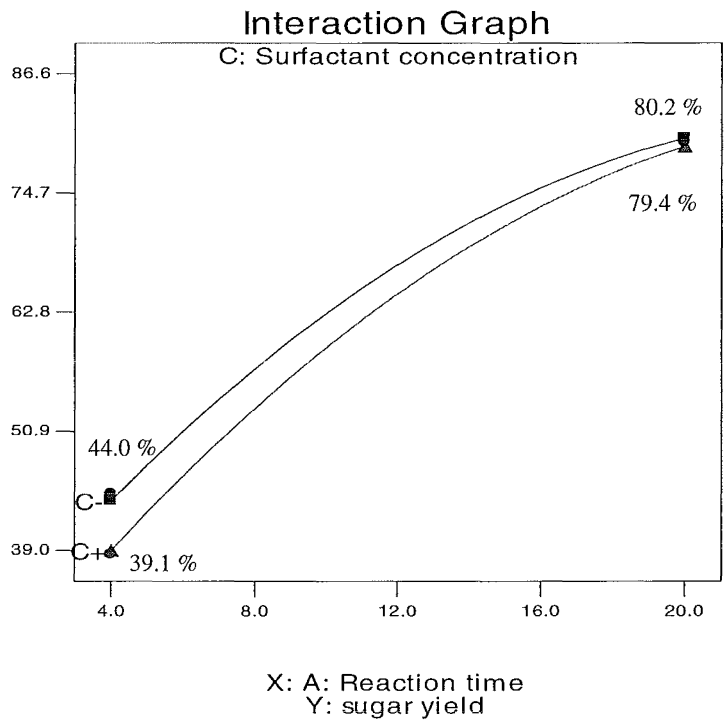
While the substrate's chemical compositions and surfactant concentrations may have differed in literature, surfactant has clearly aided the saccharification yield in other studies. However, this contradicted with the results presented in this study because most cellulase still remained active after 20 hours, even in a surfactant free condition.

A possible explanation is that office paper has a relatively lower amount of lignin compared to the other substrates mentioned above and as listed previously on **Table 2.1**. Because of this factor, the addition of surfactant up to approximately 4 g/L improved the sugar yield only marginally. Further increase would decrease the yield, probably due to product inhibition. In

other words, surfactant effect was more pronounced on substrates with high lignin content. For office paper, no significant improvement was seen.

Another possible explanation is the low recalcitrant residue on the substrate due to pretreatment with high H_3PO_4 concentration. According to Helle *et al.* (1993), surfactant addition assists in hydrolysis by modifying the substrate structure to reduce recalcitrant residue and to expose more of the surface. In this case, the pretreatment may have made it unlikely that subsequent surfactant addition had any further effect on substrate structure.

(a)



(b)

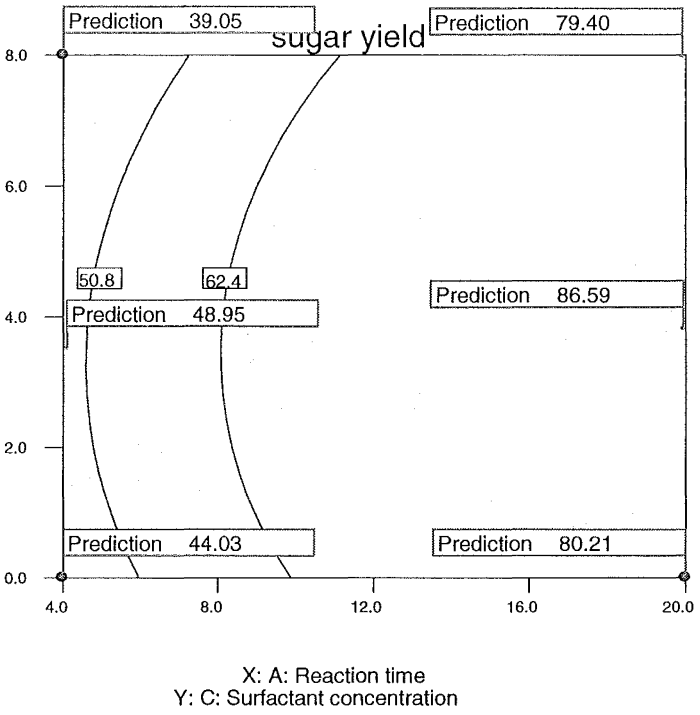


Figure 4.7 (a) Response interaction graph between reaction time and surfactant concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of reaction time and surfactant concentration

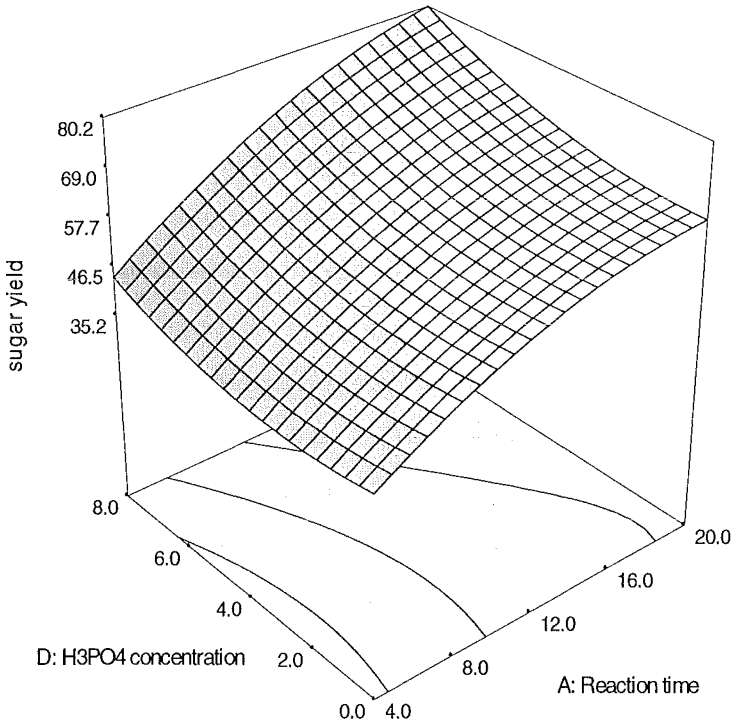


Figure 4.8 Response surface plot of sugar yield as a function of H_3PO_4 concentration and reaction time at enzyme/paper ratio (18 %) and surfactant concentration (0 g/L)

Figure 4.8 shows the effect on sugar yield of pretreatment (H_3PO_4 concentration) and reaction time with fixed enzyme/paper ratio (18 %) and surfactant concentration (0 g/L). It shows that an increase in H_3PO_4 concentration used during the pretreatment could improve yield. The hydrolysis rate also became progressively higher as H_3PO_4 concentration increased.

This is more clearly shown in **Figure 4.9 (a)** and **(b)**, where an increase in H_3PO_4 concentration from 0 g/L to 8 g/L improved the overall conversion by 27.3 % (see **Equation 4.5**) at the longest reaction time of 20 hours.

$$\left(\frac{80.2-63.0}{63.0}\right)\times 100 \qquad (4.5)$$

$$= 27.3$$

Hydrolysis could still be carried out without H_3PO_4 addition, but resulted in saturation yield when the reaction time of 20 hours was reached. With H_3PO_4 concentration at 8 g/L, the trend was also seen to be improving, but the maximum sugar yield could not be determined because the yield was still increasing at the longest reaction time and at the highest H_3PO_4 concentration.

As shown in all previous response surface plots with reaction time as a function, increased sugar yield according to reaction time was indeed an influential factor in determining the extent of digestibility. Time was required for cellulase to bind onto the insoluble substrate surface before releasing it to become soluble, reducing sugars. Unfortunately, the degradation process was slow without pretreatment. This was due to the heterogeneous structure of cellulose that contained both tightly bound, crystalline and loosely bound, amorphous constituents associated in the substrate. The most likely explanation to the decreasing yield was that as reaction progressed, more crystalline structure would be exposed, making it increasingly difficult for the large size cellulase to reach and bind to the cellulose surface. Hence, the hydrolysis was retarded towards the end of 20 hours. This theory supported the proposal from authors who described that amorphous cellulose was rapidly hydrolysed first, leaving recalcitrant cellulose unhydrolysed (Mansfield *et al.*, 1999; Koullas *et al.*, 1990; Lee and Fan, 1982). Another possible contributing factor to the declining hydrolysis rate was cellulase adsorptive loss to lignin. However, this was minimal because office paper contains relatively low lignin.

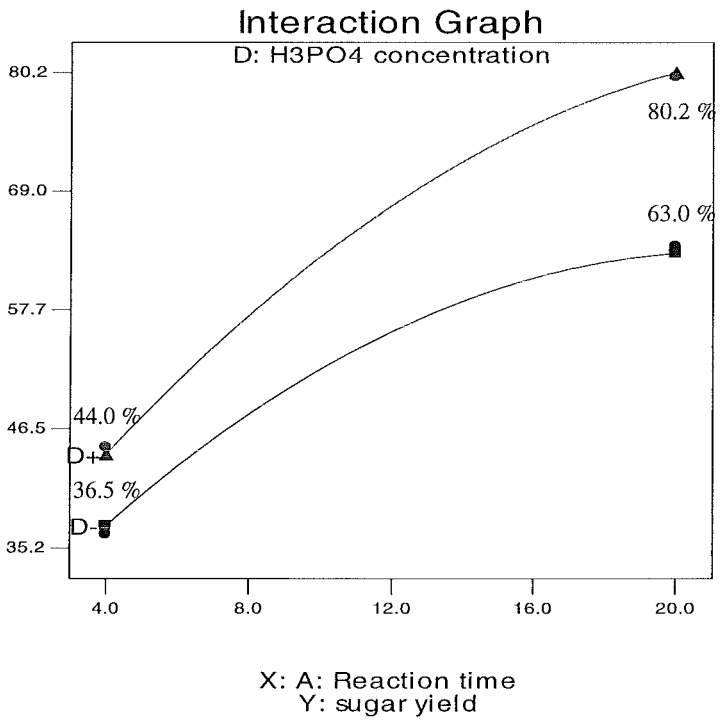
Numerous studies found that pretreatment could significantly improve enzymatic hydrolysis (Li *et al.*, 2004; Nikolov *et al.*, 2000; Zheng *et al.*, 1998; Wei *et al.*, 1996). In this study, it was

agreed that pretreatment increased the initial hydrolysis rate and assisted in improving the overall sugar yield. The extent of hydrolysis was dependent on the acid concentration used in the pretreatment. A clear correlation existed between hydrolysis yield and pretreatment.

There are speculations on the effects of pretreatment towards the disruption of the substrate's crystalline structural features. Some have demonstrated that crystalline cellulose undergoes depolymerisation, producing shorter chains of cellulose and exposing stronger interchain hydrogen bonding that gave an increase in crystallinity with increasing of hydrolysis yield (Wei *et al.*, 1996; Grethlein, 1985). Others say crystallinity was decreased after pretreatment was applied on the substrate (Zheng *et al.*, 1998), which may have enhanced the mobility of cellulase to adsorb efficiently. The actual mechanism of the crystalline structure after pretreatment still remained inconclusive. However, crystallinity may have a strong correlation with increasing surface area and/or accessibility of the cellulose surface.

Complete hydrolysis of the substrate could be obtained by extending the reaction time and it could be possible to recycle most cellulase and reuse them for subsequent enzymatic hydrolysis.

(a)



(b)

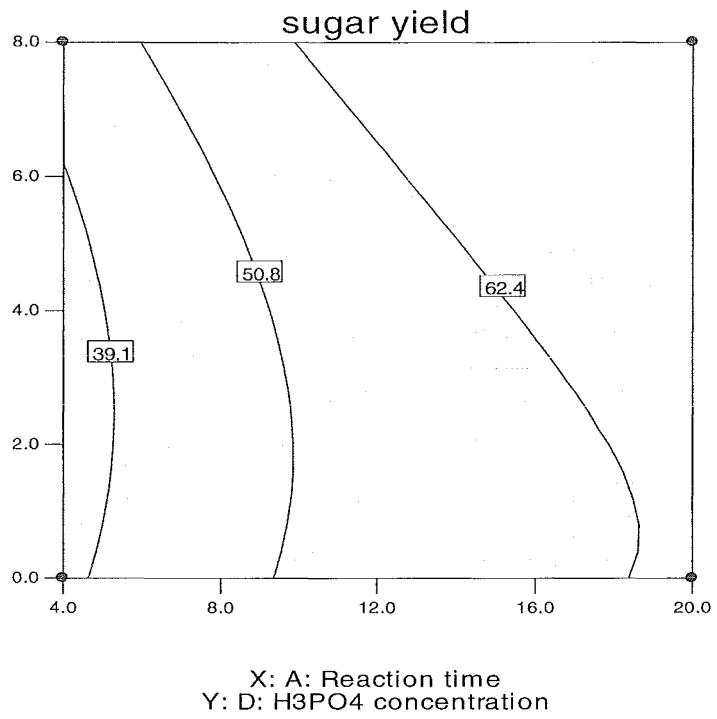


Figure 4.9 (a) Response interaction graph between reaction time and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the influence of reaction time and H_3PO_4 concentration

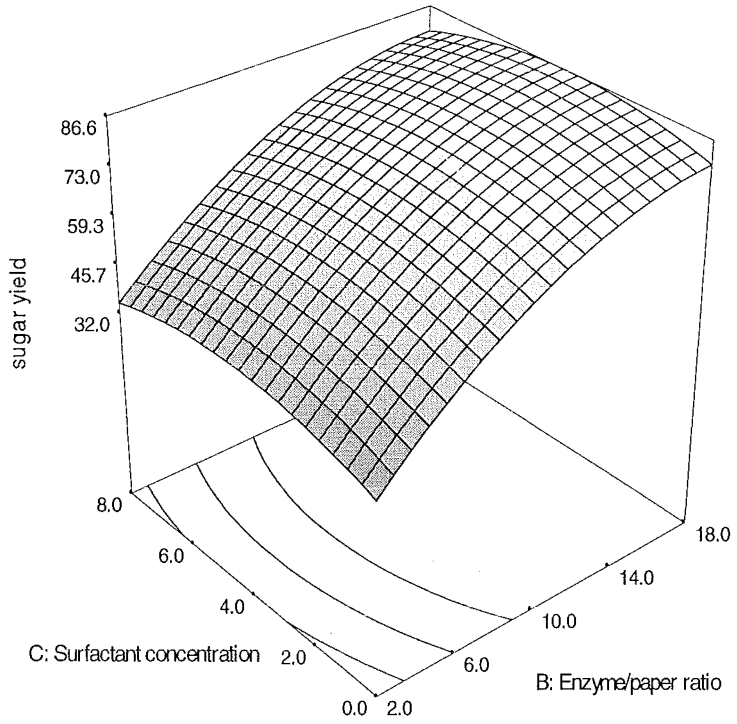


Figure 4.10 Response surface of sugar yield of as a function of surfactant concentration and enzyme/paper ratio at reaction time (20 h) and H_3PO_4 concentration (8 g/L)

Figure 4.10 shows the effect of varying surfactant concentration and enzyme/paper ratio at fixed reaction time (20 h) and H_3PO_4 concentration (8 g/L). An increase in enzyme/paper ratio was followed by an improvement in sugar yield.

From **Figure 4.11 (a)**, varying surfactant concentration at a fixed enzyme/paper ratio produced similar overall sugar yield. The maximum sugar yield, i.e. 86.6 % was obtained when surfactant concentration was close to 4 g/L, but decreased for higher and lower concentrations (see **Figure 4.9 (b)**). At minimum enzyme/paper ratio, i.e. 2 %, sugar conversion increased by 26.8 % (see **Equation 4.6**) between a surfactant free substrate and a surfactant concentration of 4 g/L, and decreased by 16.3 % (see **Equation 4.7**) between surfactant concentrations of 4 g/L and 8 g/L. At the maximum enzyme/paper ratio of 18 %, sugar conversion increased by 8 % (see **Equation 4.8**) between a surfactant free substrate and a surfactant concentration of 4 g/L, and decreased by 9.1 % (see **Equation 4.9**) between surfactant concentrations of 4 g/L and 8 g/L.

$$\left(\frac{40.7-32.1}{32.1}\right)\times 100 \qquad (4.6)$$

$$= 26.8$$

$$\left(\frac{40.7-35.0}{35.0}\right)\times 100 \qquad (4.7)$$

$$= 16.3$$

$$\left(\frac{86.6-80.2}{80.2}\right)\times 100 \qquad (4.8)$$

$$= 8.0$$

$$\left(\frac{86.6-79.4}{79.4}\right)\times 100 \qquad (4.9)$$

$$= 9.1$$

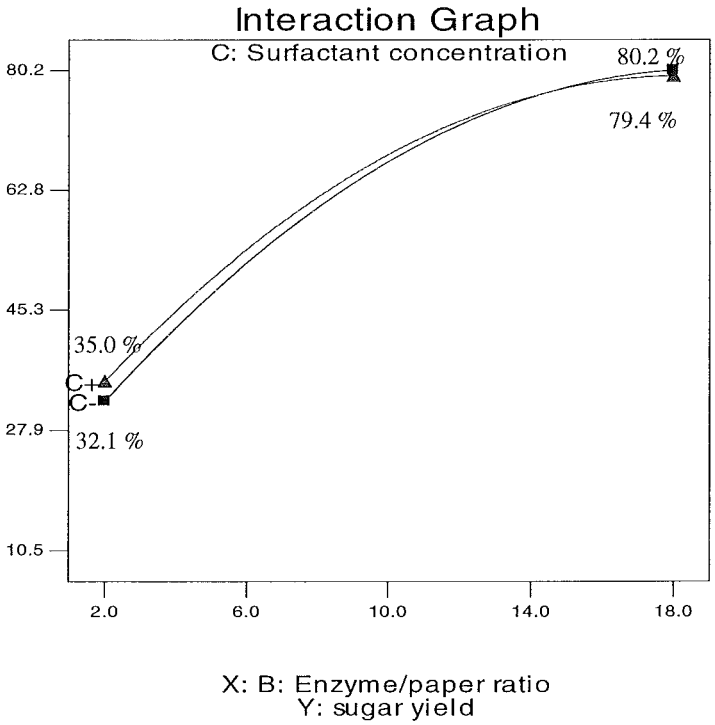
In earlier studies, Castanon and Wilke (1981) and Helle *et al.* (1993) proposed that surfactant had a substantial effect on lignocellulosic materials by preventing inactivation of cellulase on cellulose and facilitating the desorption of cellulase from the cellulose surface; thereby increasing the possibility of recycling the enzymes after completion of cellulose hydrolysis. In contrast, the presence of surfactant in this study did not appear to have much influence on

pretreated office paper with increasing enzyme/paper ratio. It was presumed in the previous section that less lignin-containing office paper meant a lesser amount of surfactant was needed to prevent unproductive binding of cellulase onto hydrophobic lignin surface. Therefore, addition of surfactant did not appear to have significant impact on the overall reducing sugars production.

Despite having negligible effect on overall sugar yield, low surfactant addition worked fairly well with low enzyme/paper ratio. A likely explanation was that minor residue of lignin was left on the outer surface of the substrate after pretreatment. When a low amount of hydrophobic cellulase was added, most of it attached to the lignin's surface before reaching the target cellulose. In this condition, surfactant worked effectively to prevent unproductive binding of cellulase on lignin. At higher enzyme/paper ratio this effect was less apparent. As cellulase concentration increased, the effectiveness of increasing surfactant addition diminished. This was not completely understood, but one possibility could be product inhibition.

The slope of both yield lines for pretreated office paper samples containing surfactant and no surfactant both remained positive at the tested maximum. This indicated that most cellulase in the solution remained active and was ready to hydrolyse more cellulose if given more time or available cellulose.

(a)



(b)

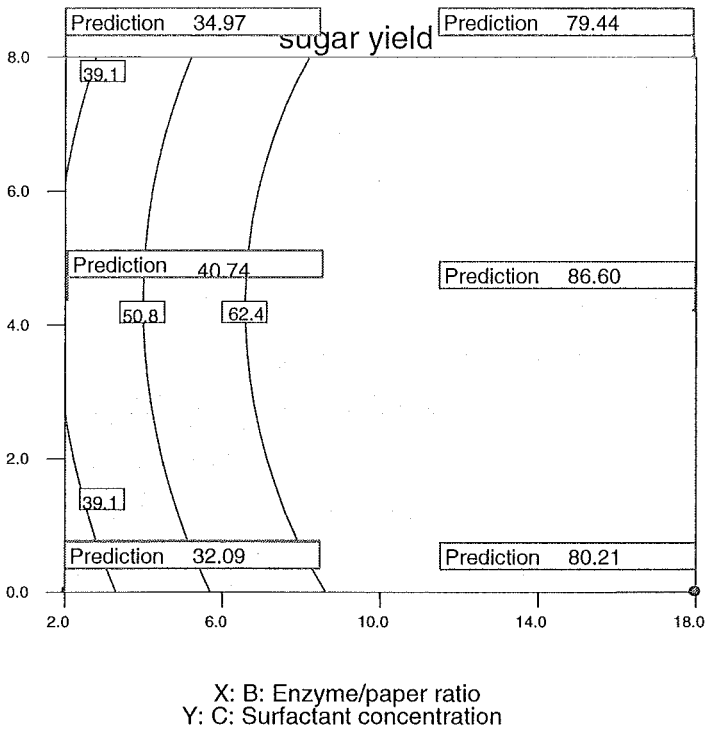


Figure 4.11 (a) Response interaction graph between enzyme/paper ratio and surfactant concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of the enzyme/paper ratio and surfactant concentration

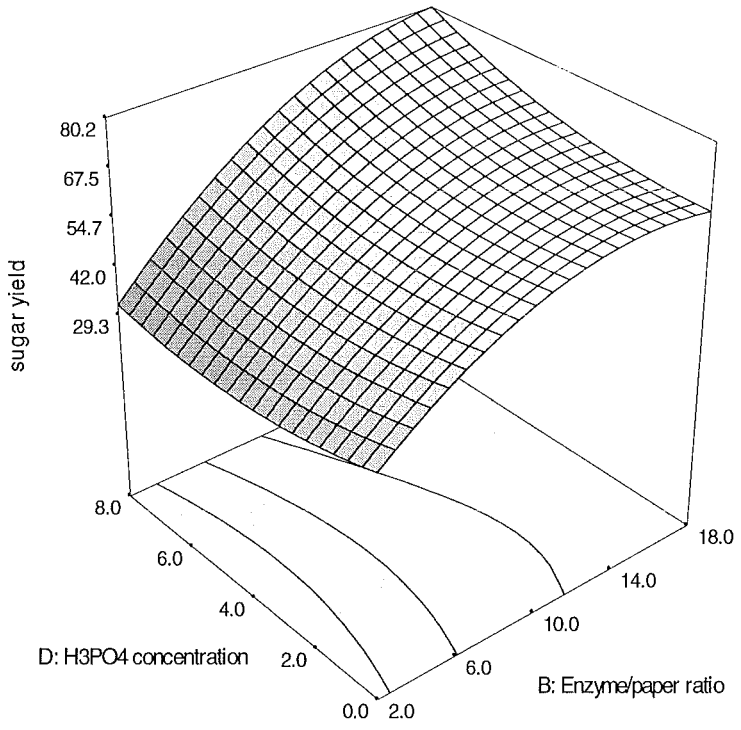


Figure 4.12 Response surface of sugar yield as a function of H_3PO_4 concentration and enzymes/paper ratio at reaction time (20 h) and surfactant concentration (0 g/L)

The effect of H_3PO_4 concentration and enzyme/paper ratio at fixed reaction time (20 h) and surfactant concentration (0 g/L) is depicted in **Figure 4.12**. The influence of H_3PO_4 and enzyme/paper ratio was apparent when concentration approached 8 g/L and enzyme/paper ratio approached 18 %.

From **Figure 4.13 (a)**, at low enzyme/paper ratio, increasing H_3PO_4 concentration had a negative effect on overall sugar yield. As enzyme/paper ratio increased, high H_3PO_4 concentrations increased the hydrolysis rate and then the rate gradually decreased towards the maximum enzyme/paper ratio. Cellulose hydrolysis without added pretreatment had 27.3 % (see **Equation 4.10**) less sugar yield compared to yield for hydrolysis with 8 g/L H_3PO_4 concentration pretreatment.

$$\left(\frac{80.2-63.0}{63.0}\right)\times 100 \qquad \textbf{(4.10)}$$

$$= 27.3$$

The optimum sugar yield for samples with no pretreatment was 64.7 % when enzyme/paper ratio reached slightly more than 14 %, as shown in **Figure 4.13 (b)**. In addition, the crossover point was found to be 6 % enzyme/paper ratio, where the overall sugar yield at 51.8 % was similar for 0 g/L and 8 g/L H_3PO_4 concentration, as shown in **Figure 4.13 (a) and (b)**.

In past and present literature, lignin and crystalline components are often known as barriers towards digestion (van Walsum and Shi, 2004; Weil *et al.*, 1994; Caulfield and Moore, 1974) because of their recalcitrant structures. It was mentioned earlier in **Section 2.3.1** that native cellulose has a crystalline component of 50-90 %. Untreated office paper has relatively low lignin content and more cellulose portions and thus, the crystalline component in office paper was considered to have the strongest influence on the extent of the conversion rate. When H_3PO_4 acid pretreatment was applied, it was believed that lignin was fractionised, resulting in partial solubilisation of the lignin. The treated office paper residue would have revealed more available surface area of lignin within the bulk of the particle than the untreated substrate. At the same time, H_3PO_4 altered the highly-ordered crystallinity structure, creating more surface area for cellulase to adsorb. When a small amount of cellulase travelled towards the substrate surface, some cellulase may have been adsorbed unspecifically to the lignin surface through hydrophobic interaction. It is to be reminded that these graphs (i.e. **Figure 4.12** and **Figure 4.13 (a)** and **(b)**) were based on a surfactant free condition. This explanation could be the most plausible reason

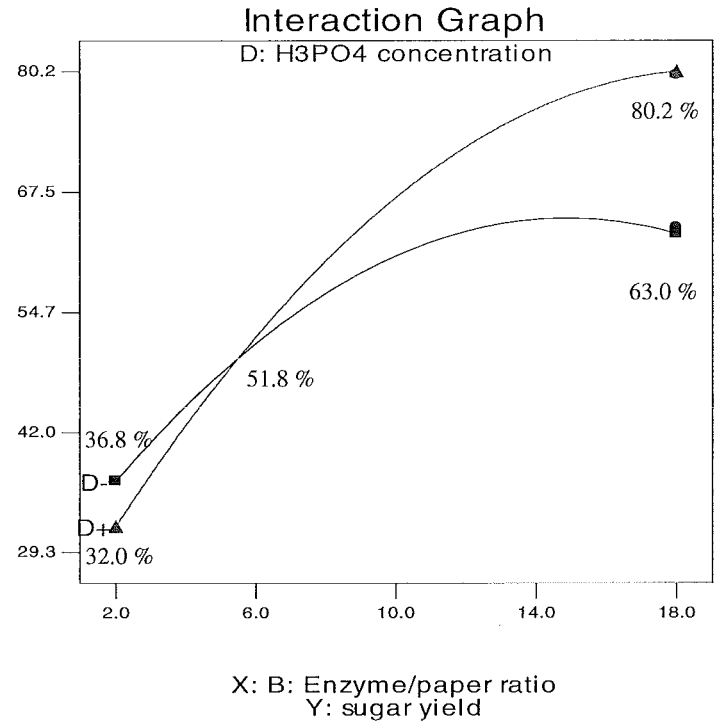
why a decrease in enzymatic hydrolysis yield was observed with maximum H_3PO_4 concentration pretreatment and at minimum enzyme/paper ratio.

Nesse *et al.* (1977) reported that steam (physical pretreatment) removed lignin without radically altering the crystallinity of the resulting cellulose, whereas Ladisch (1989) and van Wyk (1999b) proposed that acid hydrolysis (chemical pretreatment) was likely to disrupt cellulose crystallinity by destroying hydrogen bonding between crystallites and because acid molecules are about five times smaller than a cellulase enzyme, it would more readily penetrate the macroscopic structure of cellulose. Interestingly, the proposals made by these authors appeared to agree with the results currently obtained, in that the improvement of the hydrolysis was mainly due to alteration of the crystalline structure of the cellulose substrate when H_3PO_4 pretreatment was applied, but did not work so well on lignin. As a result of acid pretreatment, more lignin was thought to be exposed on the surface, compared to the untreated ones.

Enzymatic hydrolysis of pretreated substrate continued to increase with increasing enzyme/paper ratio. This could be that the available lignin binding sites were already blocked by irreversibly bound cellulase enzymes and the rest of the cellulase was free to bind specifically with cellulose. More cellulose surface area available for cellulase penetration and activity after pretreatment eventually led to a significant increase in the extent of saccharification by exceeding the rate of hydrolysis without pretreatment. Compared to treated ones, the untreated substrate samples' conversion rate slowed down due to low accessibility of the active sites for the excess cellulase to bind with.

Both pretreatment and cellulase concentration had significant effects on the degree of saccharification. Cellulose crystallinity, lignin and accessible surface area differed, depending on the substrate material. Therefore, each substrate material responded differently to various pretreatments.

(a)



(b)

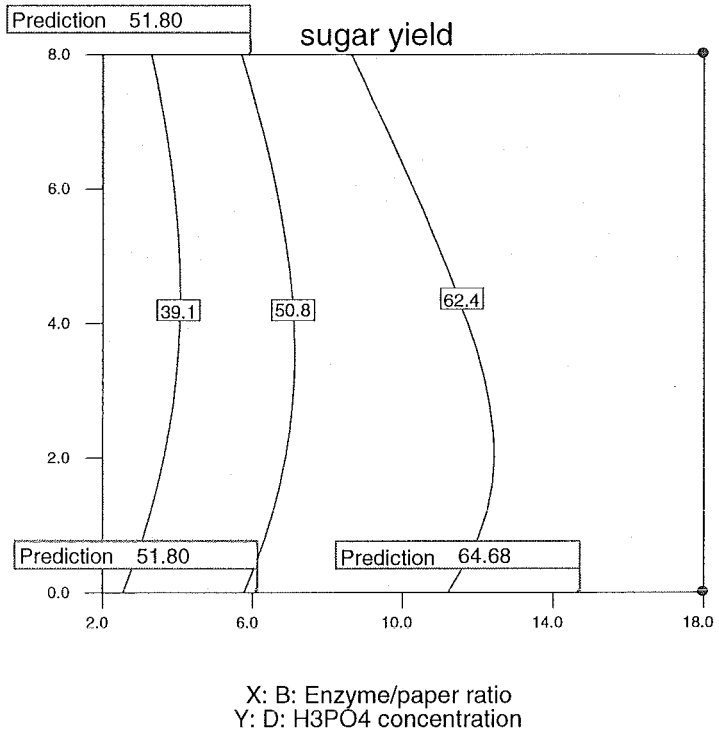


Figure 4.13 (a) Response interaction graph between enzyme/paper ratio and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of enzyme/paper ratio and H_3PO_4 concentration

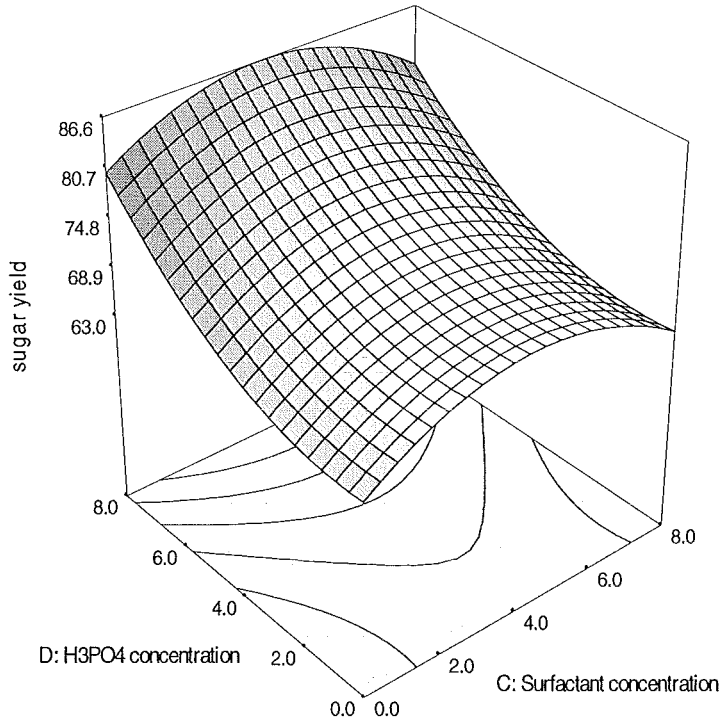


Figure 4.14 Response surface of sugar yield as a function of H_3PO_4 concentration and surfactant concentration at reaction time (20 h) and enzyme/paper ratio (18 %)

The interactive effect of H_3PO_4 concentration and surfactant concentration at fixed reaction time (20 h) and enzyme/paper ratio (18 %) is shown in **Figure 4.14**. It was observed that with surfactant addition at various H_3PO_4 concentrations, sugar yield increased up to a certain point, before the hydrolysis rate started to decrease. On the other hand, varying H_3PO_4 concentration at various surfactant concentrations showed that the initial hydrolysis rate was minimal but occurred more rapidly at higher H_3PO_4 concentration.

Figure 4.15 (a) and **(b)** illustrate that the overall conversion improved by 27.3 % (see **Equation 4.11**) when H_3PO_4 concentration increased from 0 g/L to 8 g/L.

$$\left(\frac{80.2-63.0}{63.0}\right)\times 100 \qquad \textbf{(4.11)}$$

$$= 27.3$$

At 0 g/L H_3PO_4 concentration, as surfactant concentration reached 4 g/L, the sugar yield improved by 11.9 % (see **Equation 4.12**), but decreased by 9.5 % (see **Equation 4.13**) as

surfactant increased to 8 g/L. Similarly, at 8 g/L H_3PO_4 concentration, the sugar yield improved by 7.7 % (see **Equation 4.14**) followed by a decrease of 8.8 % (see **Equation 4.15**).

$$\left(\frac{70.5-63.0}{63.0}\right)\times 100 \qquad \textbf{(4.12)}$$

$$= 11.9$$

$$\left(\frac{70.5-64.4}{64.4}\right)\times 100 \qquad \textbf{(4.13)}$$

$$= 9.5$$

$$\left(\frac{86.4-80.2}{80.2}\right)\times 100 \qquad \textbf{(4.14)}$$

$$= 7.7$$

$$\left(\frac{86.4-79.4}{79.4}\right)\times 100 \qquad \textbf{(4.15)}$$

$$= 8.8$$

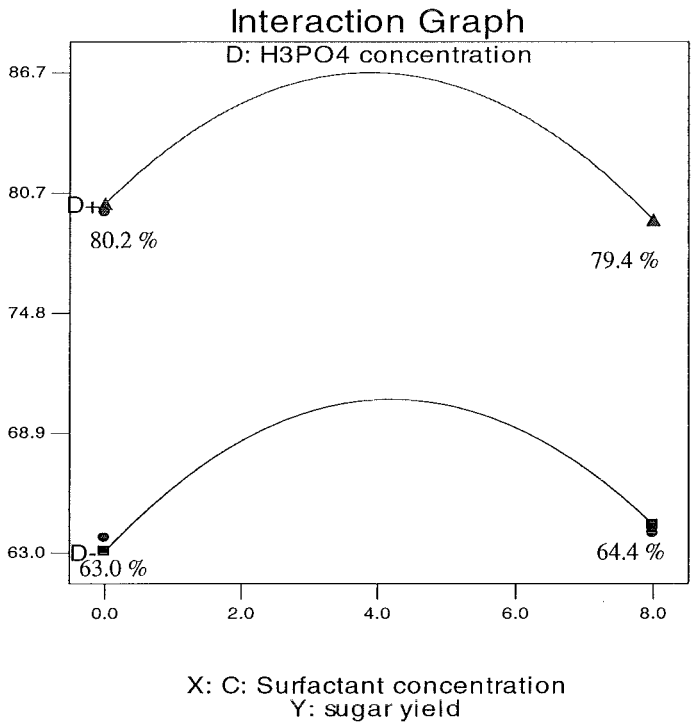
Pretreatment clearly aided in enhancement of cellulose hydrolysis by increasing the substrate surface area by altering the tightly, hydrogen bonded crystalline to let cellulase attach with more open active sites. Without pretreatment, the hydrolysis rate had a low digestibility rate because of the recalcitrance of the material. However, acid pretreatment did not appear to have removed the lignin, not even with a low lignin-containing substrate. Acid pretreatment may have resulted in revealing more of the residual lignin, which may have led to adsorption through hydrophobic interaction. Therefore, recovery of cellulase was moderately increased with surfactant addition at about 4 g/L.

These observations confirmed that pretreatment, along with reaction time and enzyme/paper ratio are three important parameters that played significant roles in enhancing sugar yield, but it was not the same case with surfactant. It was likely that lignin played an important role in preventing unspecific adsorption of cellulase on high lignin surfaces.

Interestingly, increasing surfactant concentration eventually led to a negative effect on the extent of sugar conversion. The reason for this could be product inhibition towards surfactant.

Nevertheless, more studies are needed to draw conclusions on the role of surfactant on the unspecific binding of substrate and the surfactant-cellulase relationship.

(a)



(b)

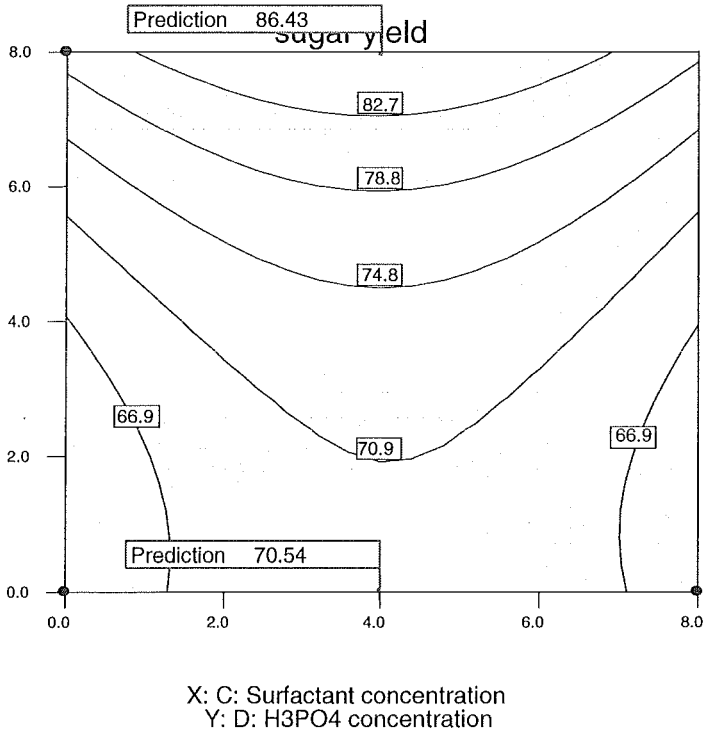


Figure 4.15 (a) Response interaction graph between surfactant concentration and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of surfactant concentration and H_3PO_4 concentration

4.1.3 Optimisation

For comparison of (a) LCRSM and (b) GAs performance, the optimum conditions of the four significant factors that yielded the maximum possible sugar conversion are listed in **Table 4.4**. The solution function file written with Matlab can be found in **Appendix D**. The results below show that both predictions in search for the optimum yield are analogous to one another. This was because the three-dimensional surface graphs obtained using *Design Expert Version 6* were relatively smooth and the optimum point could be located easily.

Table 4.4 Comparison of the optimum four factors and the maximum possible response of office paper between (a) LCRSM and (b) genetic algorithms

(a)

Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Maximum sugar yield (%)
20.0	18.0	4.2	8.0	86.6

(b)

Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Maximum sugar yield (%)
20.0	18.0	3.9	8.0	86.7

In this study, it was found that the optimisation of reaction time, enzyme/paper ratio, surfactant and H_3PO_4 pretreatment could significantly increase sugar yield. An experiment was carried out based on the optimised conditions predicted by the model to verify both the validity of the response model and its optimised conditions. It was found that it was possible to obtain relatively high sugar yield at 82.2 %, which was close to the model predicted response value.

Using LCRSM, it was possible to obtain clear information about the effects of the four factors on sugar yield. Furthermore, the experiments could be done by reducing the number of runs, time and costs for a given number of factors, compared to other experimental designs such as BBD or CCD.

4.2 Newspaper

Step 1 (Set-up and experimentation)

The experiments were performed according to the actual run order with three repeated runs as given in **Table 4.5**. The actual and predicted values of sugar yield are also given in the table.

Table 4.5 Enzymatic hydrolysis conditions, actual and predicted response variables for newspaper using LCRSM for the actual 14 run order

Run order	Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Actual sugar yield (%)	Predicted sugar yield (%)
2	20.0	18.0	0.0	8.0	13.7	13.6
9	20.0	18.0	12.0	0.0	13.9	13.9
1	8.0	2.0	3.0	8.0	6.5	6.7
11	12.0	10.0	6.0	0.0	10.8	10.5
12	16.0	6.0	9.0	6.0	12.2	11.6
10	4.0	18.0	0.0	0.0	8.2	8.4
5	12.0	10.0	0.0	4.0	5.0	4.7
4	20.0	2.0	3.0	2.0	8.6	8.9
14	16.0	6.0	9.0	6.0	11.1	11.6
7	8.0	2.0	12.0	2.0	5.6	5.8
3	4.0	18.0	12.0	8.0	5.2	5.1
8	4.0	10.0	6.0	4.0	8.0	7.7
6	12.0	18.0	6.0	4.0	10.0	10.6
13	16.0	6.0	9.0	6.0	11.7	11.6

Step 2 (Model selection)

The R^2 value of reduced model form #3 was 0.9883, which was the highest among the four selections of reduced models (refer to **Appendix C**). The sugar yield (%) in terms of Y can be written as a function of the independent variables, as represented by **Equation 4.16**.

$$Y= + 9.26 + 2.84A + 1.29B + 1.51C + 0.32D + 1.23A^2 - 0.37C^2 + 1.55D^2 + 1.19AC + 2.28AD - 0.64CD \quad (4.16)$$

where:

Y= Sugar yield (%)

A= Reaction time (h)

B= Enzyme/paper ratio (%)

C= Surfactant concentration (g/L)

D= H_3PO_4 concentration (g/L)

Step 3 (The least-squares coefficient based diagnostic)

$\beta_{q,\,est}$ was estimated using **Equation 4.17** to determine whether additional runs were required.

$$\beta_{q,\,est} = \left(\sum_{i}^{q} \beta^2_{i,est}\right)^{1/2} (q-1)^{-1/2} \qquad \textbf{(4.17)}$$

$$= [(+1.23)^2 + (-0.37)^2 + (+1.55)^2 + (+1.19)^2 + (+2.28)^2 + (-0.64)^2]^{1/2} \times (6-1)^{-1/2}$$

$$= (11.08)^{1/2} \times (5)^{-1/2}$$

$$= 1.49$$

The substitution of the second-order coefficients into **Equation 4.17** yielded a value of 1.49, which was more than $\sigma_{prediction}$. Hence, an additional four runs needed to be performed as specified previously in **Section 3.14**.

Step 4 (Additional runs)

Table 4.6 Enzymatic hydrolysis conditions, actual and predicted response variables for newspaper using LRSM for the actual 18 run order

Run order	Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Actual sugar yield (%)	Predicted sugar yield (%)
2	20.0	18.0	0.0	8.0	13.7	13.8
9	20.0	18.0	12.0	0.0	13.9	14.0
1	8.0	2.0	3.0	8.0	6.5	6.56
11	12.0	10.0	6.0	0.0	10.8	10.9
12	16.0	6.0	9.0	6.0	12.2	11.6
10	4.0	18.0	0.0	0.0	8.2	8.5
5	12.0	10.0	0.0	4.0	5.0	5.1
4	20.0	2.0	3.0	2.0	8.6	8.7
18	20.0	18.0	0.0	0.0	12.8	12.6
7	8.0	2.0	12.0	2.0	5.6	5.7
3	4.0	18.0	12.0	8.0	5.2	5.3
8	4.0	10.0	6.0	4.0	8.0	8.1
6	12.0	18.0	6.0	4.0	10.0	10.0
13	16.0	6.0	9.0	6.0	11.7	11.6
14	16.0	6.0	9.0	6.0	11.1	11.6
15	4.0	18.0	0.0	8.0	8.3	8.1
16	4.0	2.0	0.0	0.0	4.0	3.9
17	4.0	18.0	12.0	0.0	5.4	5.2

Table 4.6 shows the experimental results that were run in random order with a total of 18 runs. The Analysis of Variance (ANOVA) tables in **Appendix C** showed that among the four candidates of the reduced model forms, model form #1 had the highest R^2 value. The R^2 value was found to be 0.9557, which was reasonably good. As a result, this reduced model appeared to be adequate for predicting sugar yield.

Next, the data was reanalysed with a full quadratic model to determine whether it provided a better model than reduced model form #1 for predicting sugar yield. The R^2 value was 0.9949 (refer to **Appendix C**). The R^2 value suggested that the full model would be the most appropriate

model for predicting the response by the four independent variables at various conditions. The full model improved the R^2 value by 0.0392 through the inclusion of four additional coefficients and did not result in any increase in computational time. Furthermore, **Figure 4.16** shows that the scatter plot of actual against predicted sugar yield for the full model forms a relatively straight pattern, with an R^2 value of 0.9949. In view of this, the full model was selected.

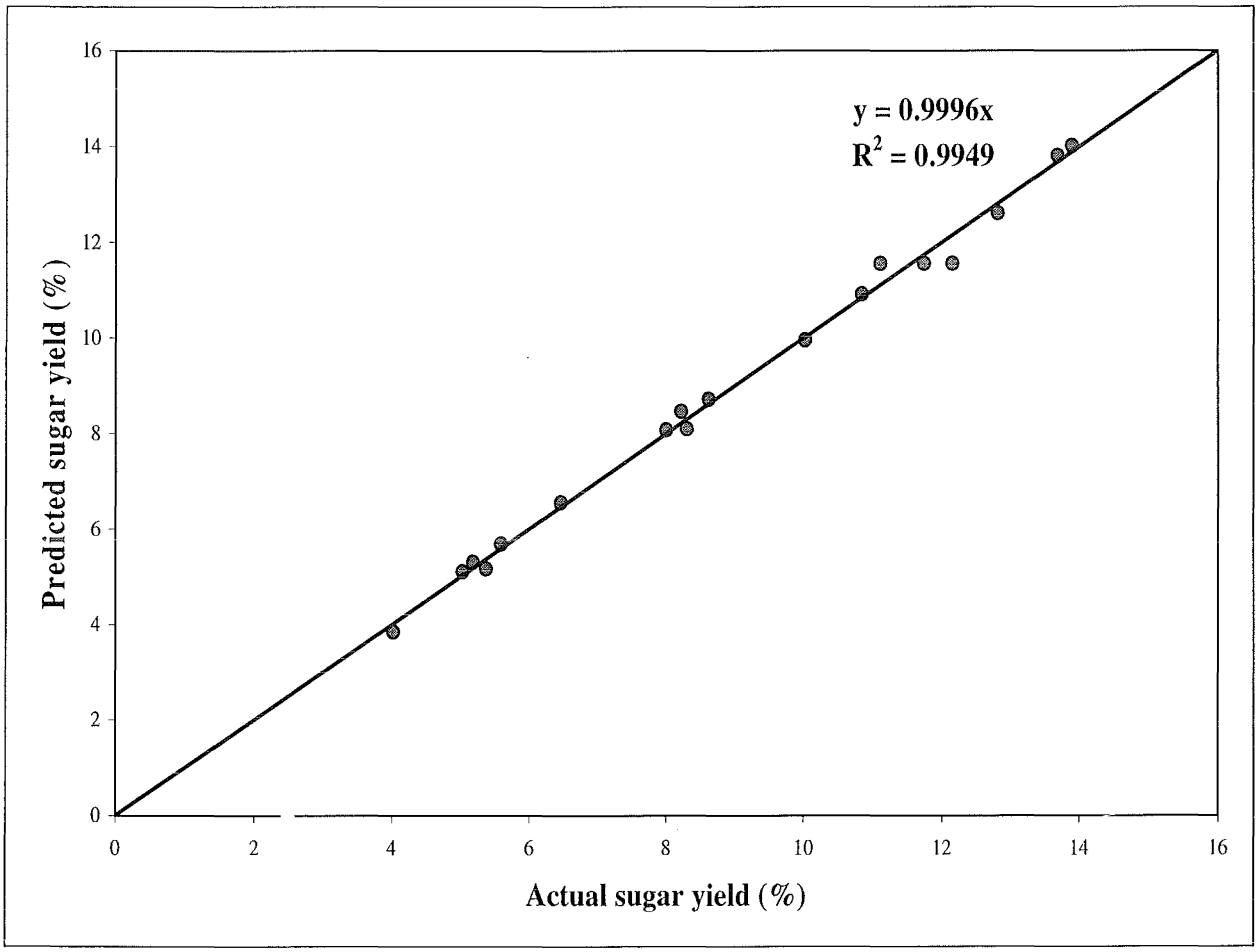


Figure 4.16 Full model plot for actual vs. predicted sugar yield

The final prediction model in terms of coded factors is given in **Equation 4.18**.

$$Y = +9.41 + 3.03A + 1.38B + 1.17C + 0.36D + 1.70A^2 - 0.84B^2 - 3.13C^2 + 1.87D^2 + 0.61AB + 1.18AC + 0.39AD - 1.51BC - 0.019BD + 0.13CD \quad (4.18)$$

With the inputs expressed in terms of actual factors, the quadratic response surface model is represented by **Equation 4.19**.

$$\begin{aligned}\text{Sugar yield} = {} & + 4.58552 - 0.5515\text{Reaction time} + 0.51196\text{Enzyme/paper ratio} \\ & + 1.23714\text{Surfactant concentration} - 1.01835\text{H}_3\text{PO}_4\text{ concentration} \\ & + 0.026615\text{Reaction time}^2 - 0.013081\text{Enzyme/paper ratio}^2 \\ & - 0.086879\text{Surfactant concentration}^2 + 0.11671\text{H}_3\text{PO}_4\text{ concentration}^2 \\ & + 0.00952488\text{Reaction time Enzyme/paper ratio} \\ & + 0.024547\text{Reaction time Surfactant concentration} \\ & + 0.012289\text{Reaction time H}_3\text{PO}_4\text{ concentration} \\ & - 0.031550\text{Enzyme/paper ratio Surfactant concentration} \\ & - 0.000606489\text{Enzyme/paper ratio H}_3\text{PO}_4\text{ concentration} \\ & + 0.00534323\text{Surfactant concentration H}_3\text{PO}_4\text{ concentration}\end{aligned} \quad \textbf{(4.19)}$$

4.2.1 Scanning Electron Microscope (SEM) Analysis

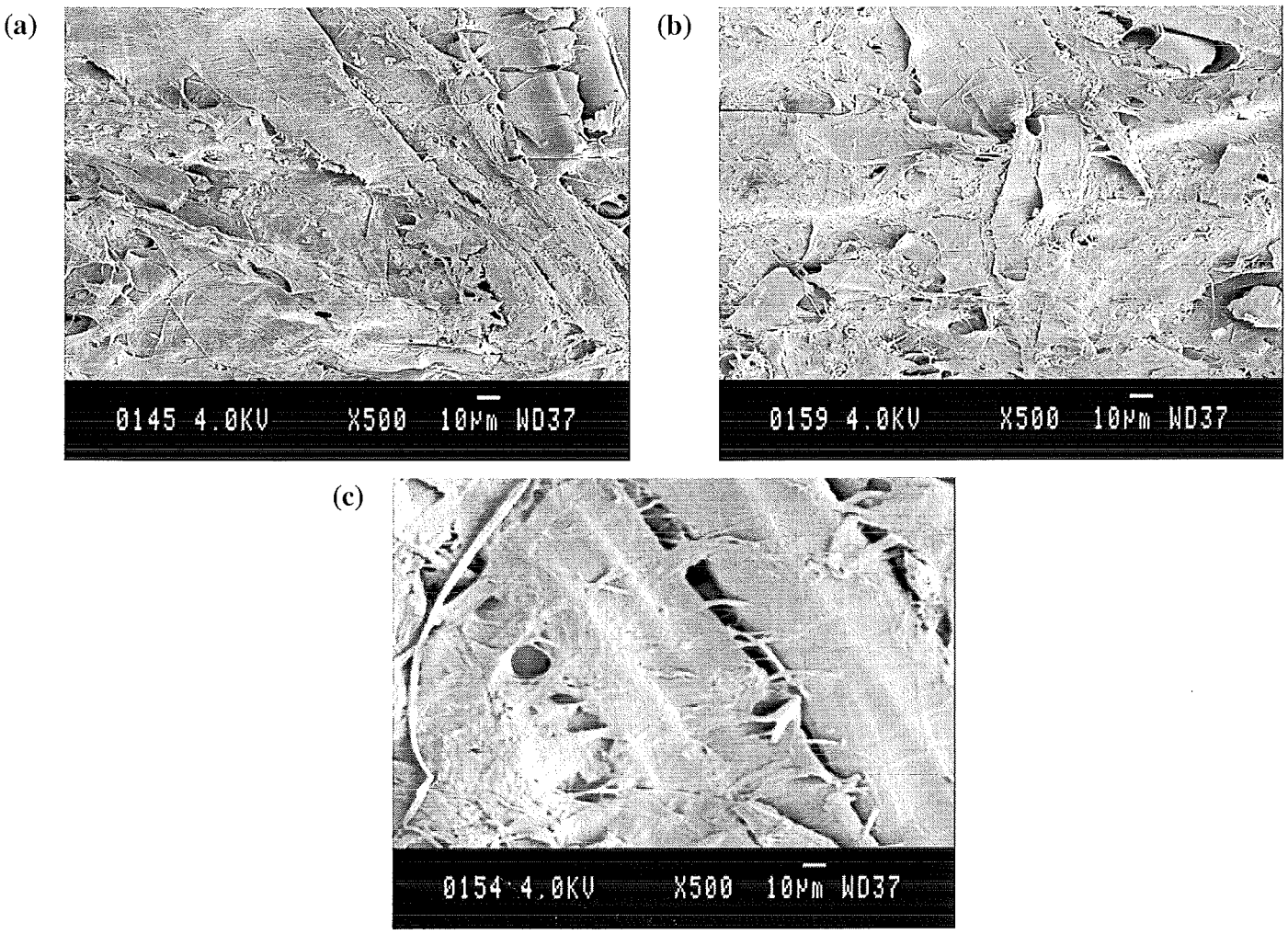


Figure 4.17 SEM-micrographs of unhydrolysed newspaper surface with (a) no H_3PO_4 pretreatment (b) 4 g/L H_3PO_4 pretreatment and (c) 8 g/L H_3PO_4 pretreatment

Figure 4.17 shows typical electron micrographs of the appearance of newspaper with and without pretreatment. With no pretreatment, all the fibers were compressed with one another such that accessible surface area for cellulase to attack appeared to be very limited. When 4 g/L H_3PO_4 pretreatment was employed, the overall fibers' dimensions could have altered to a greater extent such that the surface layers appeared to be more closely linked together, which led to an increase in the structural integrity of the fibers. The loosening effect of the fibers' segments was further seen after 8 g/L H_3PO_4 pretreatment was applied to the substrate. This suggested that more surfactant and cellulase were allowed to bind on the new binding sites and hence, an increase in hydrolysis yield could be expected.

These pictures are only meant for observation and are not integral to the discussion of the results.

4.2.2 Response Surface Plots

4.2.2.1 Influence of Four Input Factors on the Overall Sugar Yield

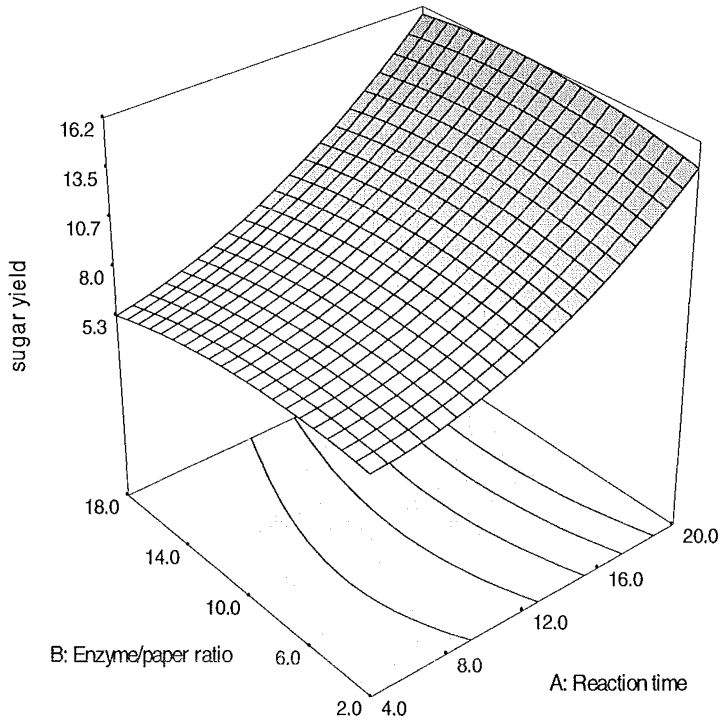


Figure 4.18 Response surface of sugar yield as a function of enzyme/paper ratio and reaction time at surfactant concentration (12 g/L) and H_3PO_4 concentration (8 g/L)

The relationship between enzyme/paper ratio and reaction time at fixed surfactant concentration (12 g/L) and H_3PO_4 concentration (8 g/L) is shown in **Figure 4.18**. At varying enzyme/paper ratio, the rate of hydrolysis was approximately similar. Varying reaction time showed that the initial rate was low, followed by a steady increase upon which a maximum of 16.2 % cellulose was converted to reducing sugars in 20 hours.

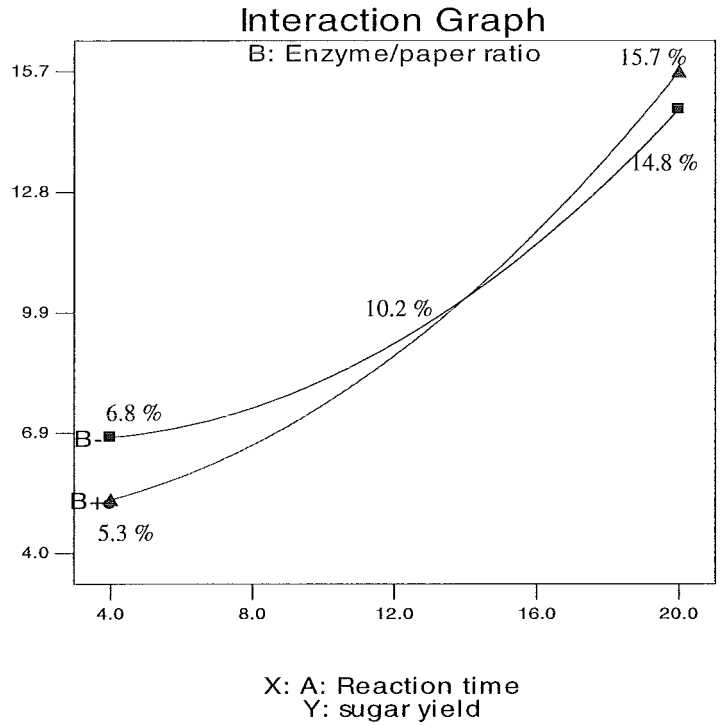
At the shortest reaction time, a slightly higher sugar conversion was observed at the lowest enzyme/paper ratio, i.e. 2 % as compared to the highest enzyme/paper ratio at 18 %, with a sugar yield of 6.8 % and 5.3 % respectively, as shown in **Figure 4.19 (a)**. At 14 hours, the rate of hydrolysis using different enzyme/paper ratio was consistent, with 10.2 % cellulose conversion, as shown in **Figure 4.19 (a)** and **(b)**. The conversion rate continued to increase after prolonged reaction time. At 20 hours, the sugar yield reached 14.8 % and 15.7 % at 2 % and 18 % enzyme/paper ratio respectively. It appeared that cellulose conversion remained active after the

maximum reaction time at 20 hours and enzyme/paper ratio was not a major contributor to overall cellulose conversion.

The enzymatic hydrolysis of newspaper was extremely low compared to office paper. The cellulase used to hydrolyse office paper and newspaper in this work had different hydrolytic activities. There were 8.5 units present in the 5 ml solution in the office paper experiments and 8.1 units present in the 5 ml solution in the newspaper experiments (see **Section 3.2**). This would be equivalent to 1.7 units/ml and 1.62 units/ml for office paper and newspaper respectively. This difference is, however, comparatively small and would not produce such huge differences in the rate and extent of hydrolysis between both substrates. Consequently, other factors would be at least partly responsible for this poorly achieved sugar yield. These factors may have included:

1. Substrate heterogeneous feature: Newspaper is rich in lignin (see **Table 2.1**), which is a recalcitrant material that prevents cellulase from degrading cellulose (Sun and Cheng, 2002; Gregg and Saddler, 1996). Most cellulose remained unsuitable for bioconversion unless lignin was removed or modified.
2. Accessible surface area: Another factor that could influence the hydrolysis rate was accessibility. Poor access to the substrate limited the amount of cellulase that could come into effective contact with cellulose.
3. Surfactant: It was believed that surfactant controlled unspecific adsorption of cellulase to the substrate and optimisation for lignocellulosics would contribute further to efficient hydrolysis. **Figure 4.18** and **Figure 4.19 (a)** and **(b)** are based on maximum surfactant concentration employed at 12 g/L, but the yield did not seem to have improved significantly with the addition of surfactant. It could be that the surfactant concentration may be too low to have any effects with high lignin-containing substrate.
4. Pretreatment: Even after pretreatment, most lignin could have remained rigid and intact with cellulose.

(a)



(b)

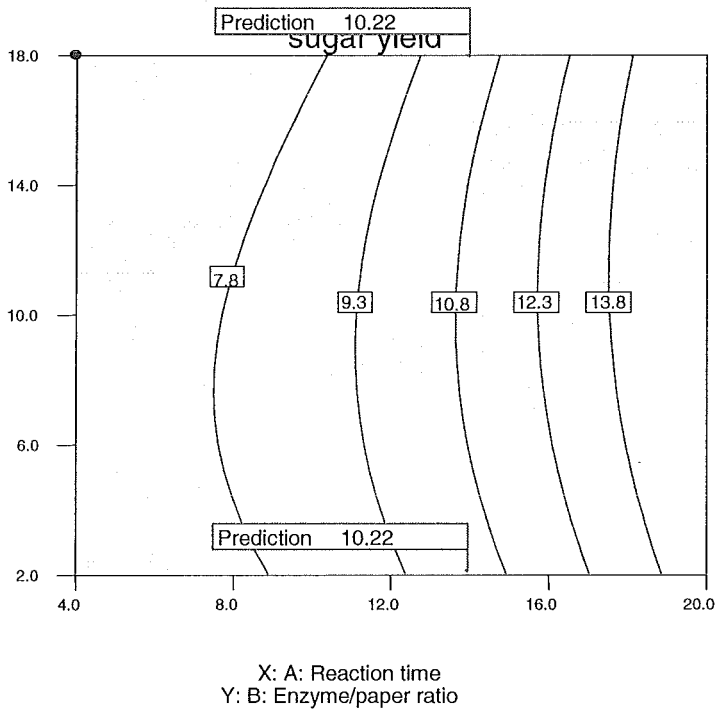


Figure 4.19 (a) Response interaction graph between reaction time and enzyme/paper ratio at (▲) 18 % and (■) 2 % and (b) response contours between the interaction of reaction time and enzyme/paper ratio

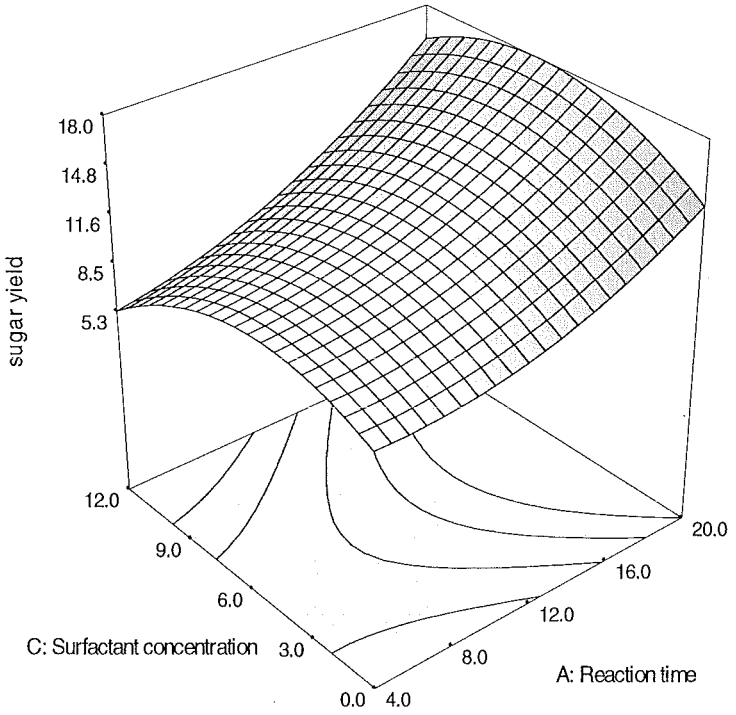


Figure 4.20 Response surface of sugar yield as a function of surfactant concentration and reaction time at enzyme/paper ratio (18 %) and H_3PO_4 concentration (8 g/L)

Figure 4.20 demonstrates the reaction between surfactant concentration and reaction time at fixed enzyme/paper ratio (18 %) and H_3PO_4 concentration (8 g/L). It was clearly seen that the maximum yield could be obtained when surfactant concentration reached an optimum point. Sugar yield also increased with increasing reaction time, regardless of varying surfactant concentration.

Figure 4.21 (**a**) and (**b**) show that low surfactant concentration addition could be beneficial to cellulose hydrolysis at a short reaction time. For example, consider the following for a reaction time of 4 hours. In a surfactant free condition, the sugar yield achieved was 8.1 %. At a surfactant concentration of 12 %, the sugar yield was 5.3 %. This meant that the addition of surfactant concentration to 12 % decreased sugar yield by 52.8 %, as shown in **Equation 4.20**.

$$\left(\frac{8.1-5.3}{5.3}\right)\times 100$$

$$= 52.8 \qquad \textbf{(4.20)}$$

When reaction time reached approximately 14 hours, surfactant concentration at 12 % resulted in a similar response to the surfactant free treatment, i.e. a 10.1 % sugar yield. Extending the

reaction time up to 20 hours revealed that surfactant concentration at 12 % allowed cellulose hydrolysis to reach 15.7 % compared to 13.8 % for a surfactant free condition.

Finally, the best response was achieved with surfactant concentration close to 6.8 g/L, where the sugar yield reached 18 % at 20 hours, as shown in **Figure 4.21 (b)**.

Previously, researchers have postulated that increasing surfactant loading could improve newspaper saccharification yield as it assisted in desorption of cellulase from cellulose (Park *et al.*, 1992; Castanon and Wilke, 1981). Based on this study, the positive effect of surfactant addition was seen with newspaper up to approximately 6.8 g/L, above which further enhancement would only have a negative effect. This was probably due to the different substrate used in the studies. Both Park *et al.* (1992) and Castanon and Wilke (1982) prepared their substrate in suspension form instead of solid substrate. According to Walker (1993), lignocellulosic materials swell when it gained moisture and lost it once it shrinks, which meant the void spaces in between the fiber network were smaller for dry substrates. Kim and Hong (2001) conducted a set of experiments between dry-pretreated wood and moisture-pretreated wood samples and discovered that substrate containing moisture resulted in high reducing sugars yield whereas for dry-pretreated wood, digestibility remained almost the same as native wood samples.

For this study, the lignin surface may have been redistributed and exposed after acid pretreatment. Initial surfactant addition decreased unspecific adsorption of cellulase by attaching onto the exposed lignin surface area. However, as more surfactant was added, the excess surfactant was unable to bind with the rest of the lignin that was still tightly cross-linked with cellulose. Hence, the extent of surfactant addition was very much dependent on the amount of lignin exposed on the outer surface.

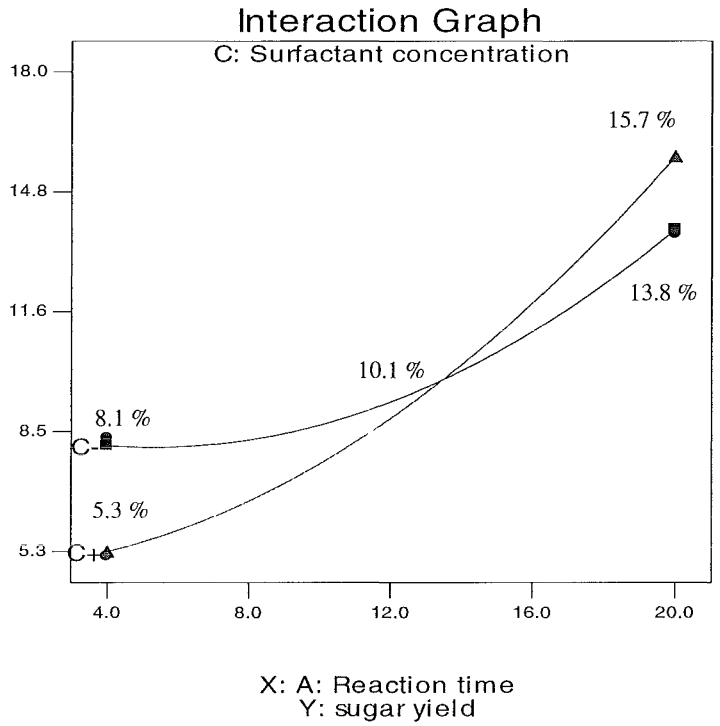
Surfactant and cellulase competed with each other to attach to the substrate surface. In hydrolysis experiments, surfactant was added before addition of cellulase. Referring to **Figure 4.21 (a)**, pretreated substrate coated with 12 g/L surfactant including the excess in the solution could have initially prevented some cellulase from passing through the layer of surfactant. As reaction time continued, more and more cellulase was able to pass through the accessible area and bind specifically to the active sites. For pretreated substrate without a layer of surfactant coating however, high cellulase concentration (i.e. 18 %) would bind specifically and unspecifically with the cellulose surface. As time passed, lesser amounts of cellulase could be recovered (i.e. 6.8 %)

because some were adsorbed irreversibly to the lignin surface. Therefore, the yield line for maximum surfactant concentration was above the yield line without surfactant condition at the end of 20 hours.

The optimum surfactant concentration was about 6.8 g/L, at which the yield was highest among all surfactant concentrations at varying reaction time. This may be because at this concentration, the surfactant may have been just adequate to form a thin layer that attached to lignin while still allowing cellulase to pass through to the active sites.

Enzymatic hydrolysis continued to release more reducing sugars until all accessible cellulose was fully degraded by the cellulase. With this, the rate and the extent of hydrolysis increased with extending reaction time before a declining yield could be observed.

(a)



(b)

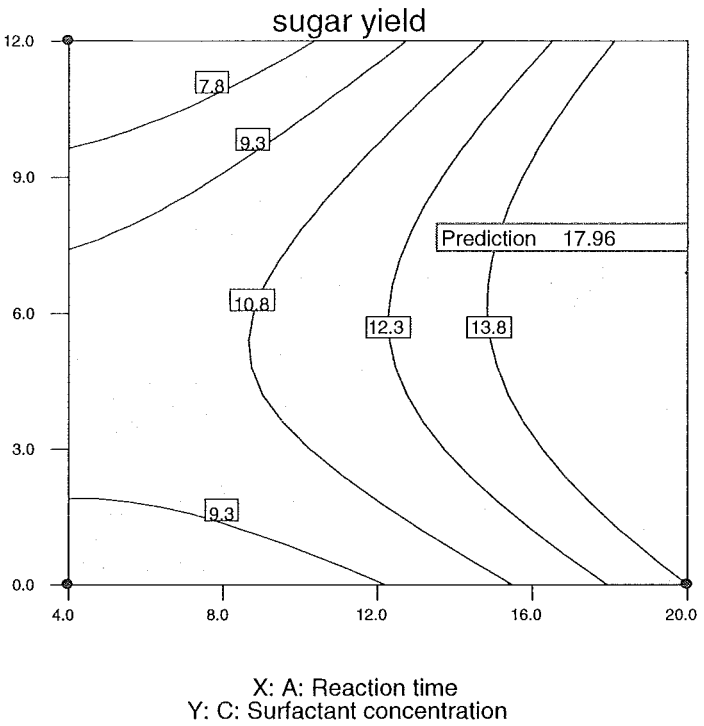


Figure 4.21 (a) Response interaction graph between reaction time and surfactant concentration at (▲) 12 g/L and (■) 0 g/L and (b) response contours between the interaction of reaction time and surfactant concentration

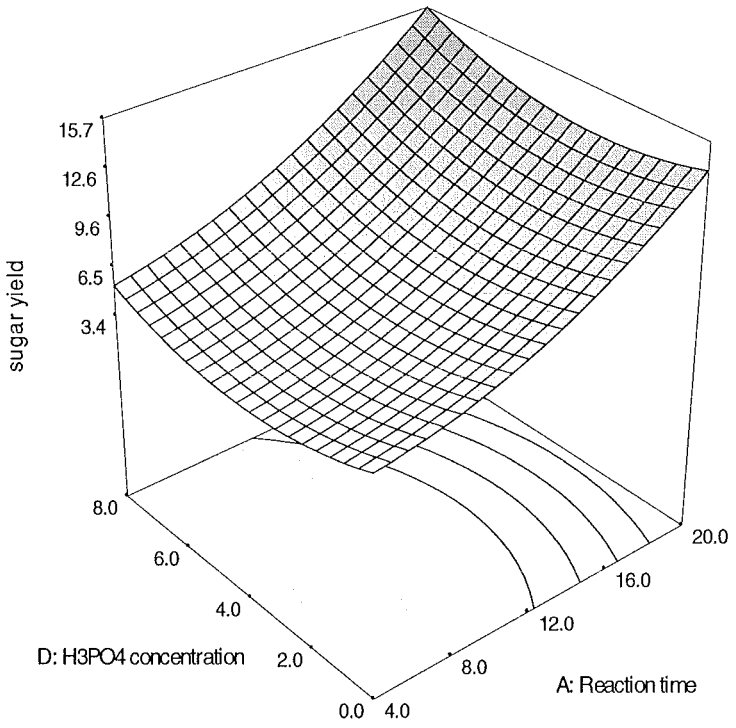


Figure 4.22 Response surface plot of sugar yield as a function of H_3PO_4 concentration and reaction time at enzyme/paper ratio (18 %) and surfactant concentration (12 g/L)

The effect of H_3PO_4 concentration and reaction time at fixed enzyme/paper ratio (18 %) and surfactant concentration (12 %) is shown in **Figure 4.22**. Sugar yield gradually increased with an increase in reaction time up to 20 hours. On the other hand, sugar yield decreased with an increase in H_3PO_4 concentration up to approximately 4 g/L and then gradually increased.

When reaction time was at 4 hours, the increment of cellulose hydrolysis was minimal between substrate without pretreatment and substrate pretreated with H_3PO_4 concentration up to 8 g/L, as represented in **Figure 4.23 (a)** and **(b)**. Both have approximately the same initial rate of hydrolysis. Sugar yield reached a maximum hydrolysis conversion rate of 15.7 % and 14 % for 8 g/L and 0 g/L H_3PO_4 concentration respectively in 20 hours. This was equivalent to an increased digestibility rate of 12.1 % (see **Equation 4.21**).

$$\left(\frac{15.7-14.0}{14.0}\right)\times 100$$

$$= 12.1 \qquad \textbf{(4.21)}$$

As shown in previous interaction graphs, the hydrolysis rate showed a positive trend as reaction time continued, regardless of varying amounts of H_3PO_4. For example, **Figure 4.23 (b)** shows that H_3PO_4 concentration close to 4 g/L has the lowest sugar yield compared to 0 g/L and 8 g/L. Still, the hydrolysis rate improved significantly when time reached 20 hours with an increase in the extent of digestibility up to 282.4 % (see **Equation 4.22**).

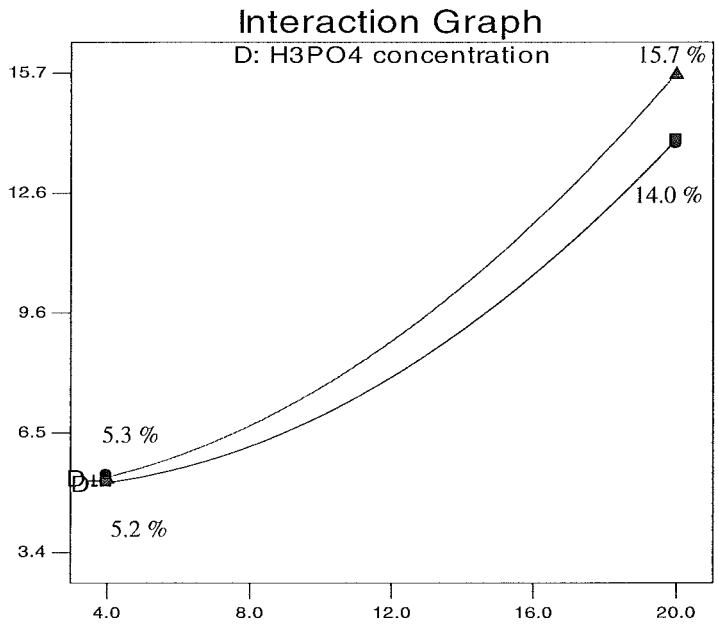
$$\left(\frac{13.0-3.4}{3.4}\right)\times 100$$

$$= 282.4 \qquad \textbf{(4.22)}$$

The structural and compositional factors (i.e. lignin, hemicellulose and crystallinity) for lignocellulosic materials often affect the susceptibility of cellulase attack. According to our previous study, increasing dilute H_3PO_4 pretreatment on office paper gave minimal solubilisation of lignin, but was able to alter the crystalline structure of cellulose that led to increased digestibility. For high lignin-containing substrate such as newspaper, it was expected that the lignin remained largely undisrupted by H_3PO_4 but altered the crystallinity and solubilised hemicellulose increased the surface area to a certain extent.

At 4 g/L H_3PO_4 concentration however, it was observed that the overall yield line was lower compared to the untreated samples. It is most probable that when pretreatment was applied, some lignin was altered and recondensed, thus forming a more rigid and closed network that protected the cellulose from environmental exposure. This resulted in a smaller surface area than untreated samples and consequently affected the susceptibility of cellulose to enzymatic attack.

(a)



(b)

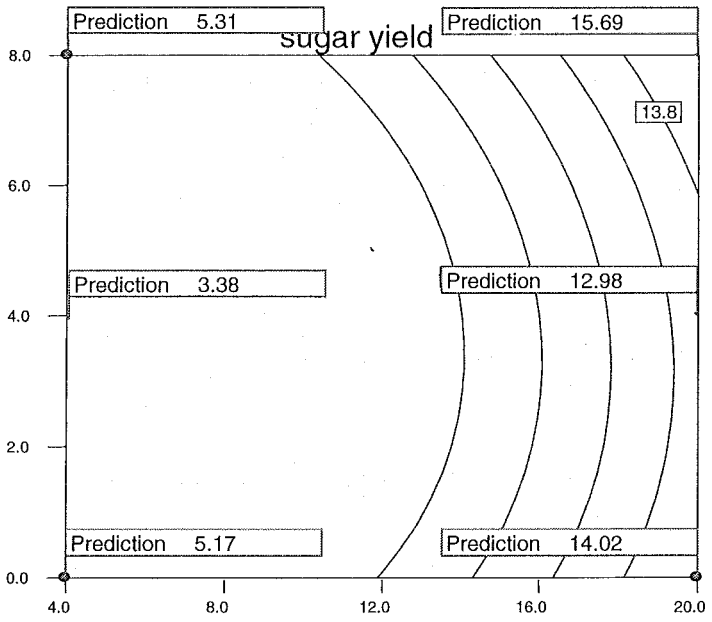


Figure 4.23 (a) Response interaction graph between reaction time and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the influence of reaction time and H_3PO_4 concentration

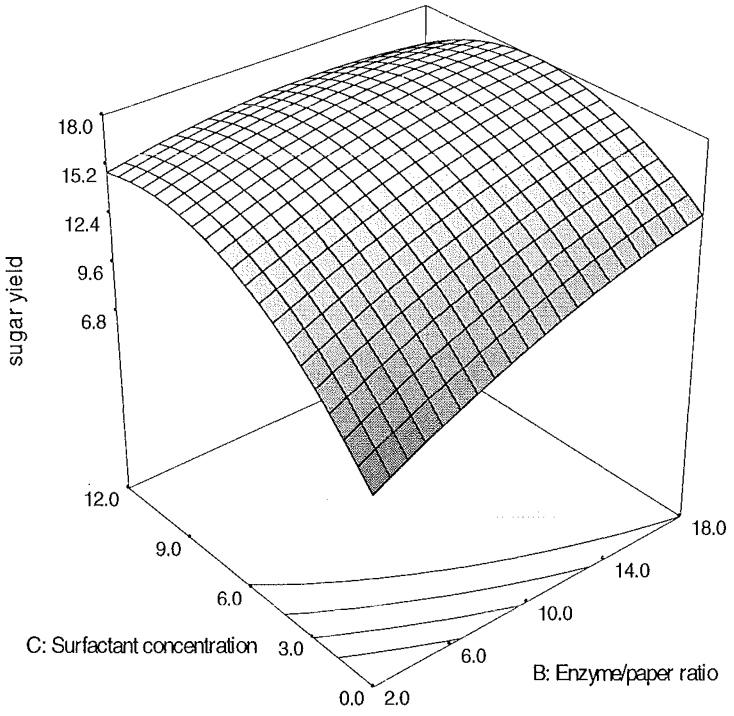


Figure 4.24 Response surface of sugar yield as a function of surfactant concentration and enzyme/paper ratio at reaction time (20 h) and H_3PO_4 concentration (8 g/L)

The effect of surfactant concentration and enzyme/paper ratio were investigated by fixing reaction time (20 h) and H_3PO_4 concentration (8 g/L), as shown in **Figure 4.24**. It was observed that an increase of surfactant concentration would increase the cellulose hydrolysis to a maximum, followed by a gradual decrease. The hydrolysis of cellulose in a surfactant free condition occurred rapidly with increasing enzyme/paper ratio. At a surfactant concentration of 12 g/L, sugar yield was found to be relatively similar at varying enzyme/paper ratio.

Figure 4.25 (a) and **(b)** again show that cellulose hydrolysis for surfactant free samples was lower than with surfactant concentration at 12 g/L for increasing enzyme/paper ratio. At maximum surfactant concentration, the conversion rate was approximately the same for increasing enzyme/paper ratio.

The maximum conversion for 12 g/L surfactant concentration was 14.8 % at 2 % enzyme/paper ratio, whereas if surfactant was not used, the maximum conversion only reached 13.8 % at 18 % enzyme paper ratio.

A similar observation was obtained for maximum surfactant concentration and enzyme/paper ratio when sugar yield was found to be 15.7 %, compared to only 13.8 % in a surfactant free condition.

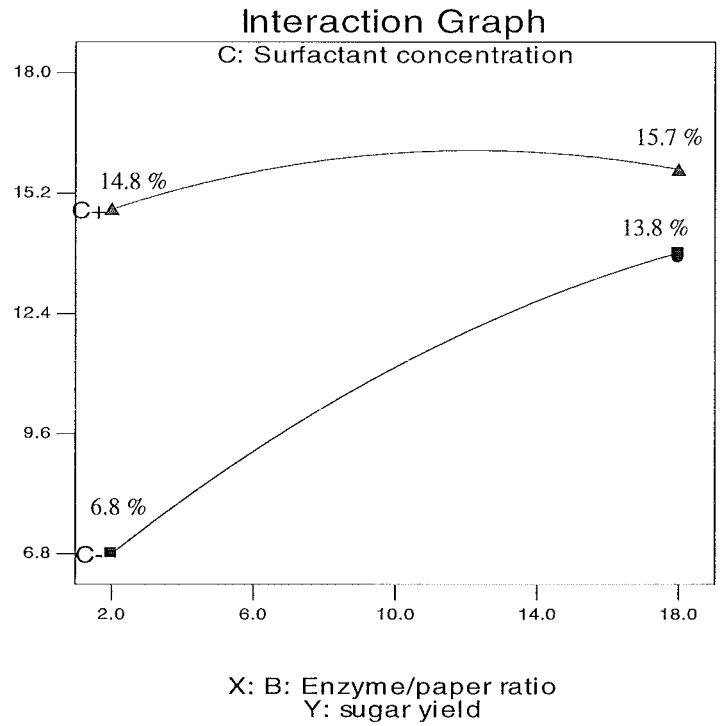
As shown in **Figure 4.25 (b)**, the optimum surfactant concentration that produced maximum sugar yield occurred around 6.8 g/L and at 18 % enzyme/paper ratio. An 18 % sugar yield was obtained at this point. Beyond this, a decrease in sugar yield was observed.

Increasing cellulase loading increased the yield to a certain extent, but would definitely increase overall process cost. This could be observed from the yield line without adding surfactant. Cellulase activity decreased during hydrolysis because of the irreversible binding of cellulase onto the cellulose surface, which was partly responsible for the low sugar yield obtained. Addition of surfactant decreased the attachment of cellulase onto the lignin surface. Unspecific adsorption of cellulase on cellulose had a much greater influence on high lignin surfaces, especially after acid pretreatment was applied.

Significant surfactant effectiveness was observed at low enzyme/paper ratio. At maximum enzyme/paper ratio, the increase in sugar yield was minimal. Inaccessibility of the cellulase was thought to be one of the major players in enhancing hydrolysis. Most extraneous substances such as lignin and hemicellulose were still in close association with cellulose. With the altered crystalline cellulose revealing its surface after pretreatment, the cellulase degraded the substrate and increased its saccharification yield. However, the hydrolysis rate was minimal because the available binding sites were still very limited. Thus, cellulase that was not attached to a specific surface area was still free in the supernatant.

It was mentioned in the previous section that the optimum surfactant concentration for efficient hydrolysis was close to 6.8 g/L and therefore, the highest yield was obtained with that concentration. Still, the hydrolysis rate was extremely low compared to office paper sugar yield. Therefore, lignin content and accessible area were more influential factors than other characteristics such as crystallinity for newspaper in determining the degree of hydrolysis.

(a)



(b)

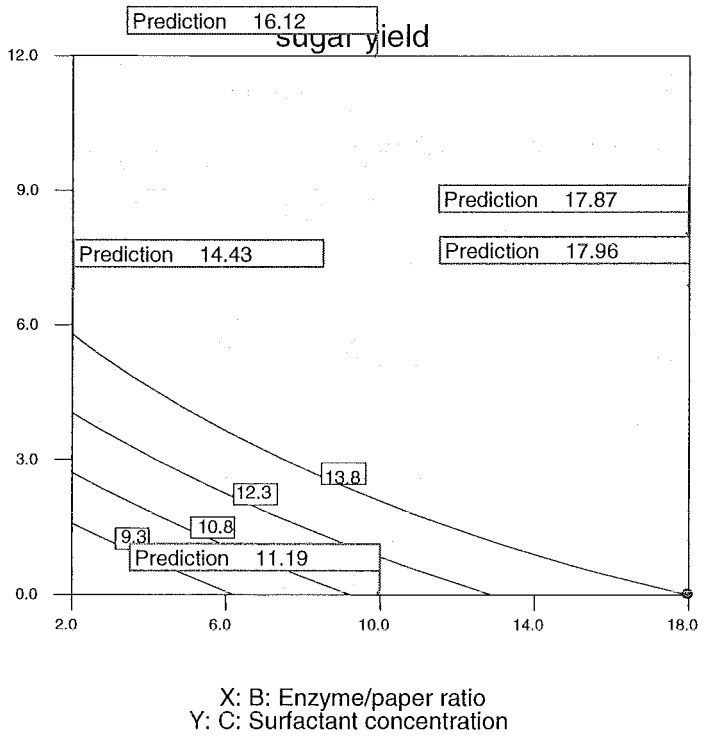


Figure 4.25 (a) Response interaction graph between enzyme/paper ratio and surfactant concentration at (▲) 12 g/L and (■) 0 g/L and (b) response contours between the interaction of the enzyme/paper ratio and surfactant concentration

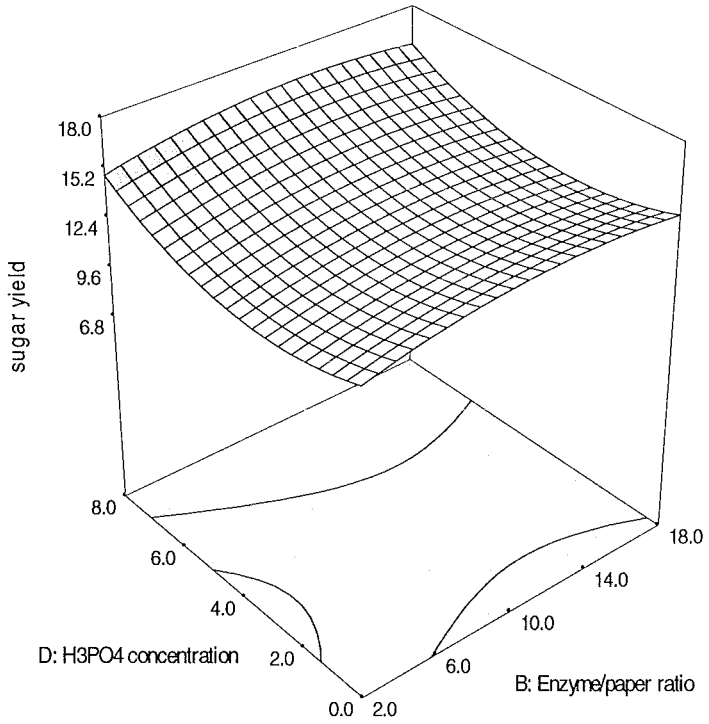


Figure 4.26 Response surface of sugar yield as a function of H_3PO_4 concentration and enzyme/paper ratio at reaction time (20 h) and surfactant concentration (12 g/L)

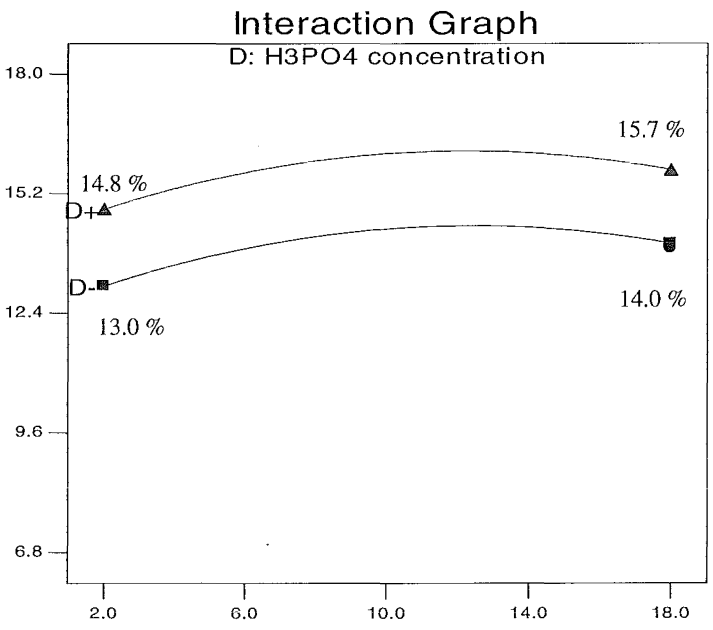
Figure 4.26 shows the interaction between H_3PO_4 concentration and enzyme/paper ratio at fixed reaction time (20 h) and surfactant concentration (12 g/L). As seen in the figure, enzyme/paper ratio did not have much influence on the hydrolysis rate. On the other hand, pretreatment of samples with H_3PO_4 clearly aided the hydrolysis rate and the extent of conversion at maximum H_3PO_4 concentration was higher than samples without pretreatment. However, pretreated samples were slightly inhibited at H_3PO_4 concentrations between 2 g/L to 4 g/L, as shown in **Figure 4.27 (a)** and **(b)**.

Figure 4.27 (a) and **(b)** show that pretreatment improved the hydrolysis rate from 13 % sugar yield at no pretreatment to 14.8 % at 8 g/L H_3PO_4 concentration when enzyme/paper ratio was 2 %.

Increasing enzyme/paper ratio up to 12 % during hydrolysis showed slightly better performance for both pretreated and untreated samples. When enzyme/paper ratio reached 18 %, both slopes showed similar trends with a decrease in sugar yield. Even so, pretreated samples at the maximum H_3PO_4 concentration still outperformed the samples without pretreatment.

The presence of lignin prevented cellulase from reaching cellulose. In addition, lignin also adsorbed cellulase, making them unavailable for cellulose hydrolysis (Eriksson et al., 2002). To increase the digestibility of lignocellulosics, pretreatment had to be applied to the substrate before it was exposed to cellulase. Exposure of H_3PO_4 pretreatment on office paper was believed to have improved the digestibility efficiency by altering the crystalline structure, thereby increasing the accessibility of cellulose to cellulase. It was expected that H_3PO_4 pretreatment on newspaper would also reduce the crystalline portion, but chemical pretreatment may not have worked efficiently on lignin surfaces, leaving a large portion of lignin intact with cellulose. Although the internal surface area had increased and the outer surface may have slightly revealed its weaker spots, most cellulase was still unable to penetrate through and bind effectively to the cellulose. The extent of hydrolysis was much more dependent on the distribution and composition component of newspaper. Therefore, the residual substrate following hydrolysis for both pretreated and non-pretreated yield lines was very similar.

(a)



(b)

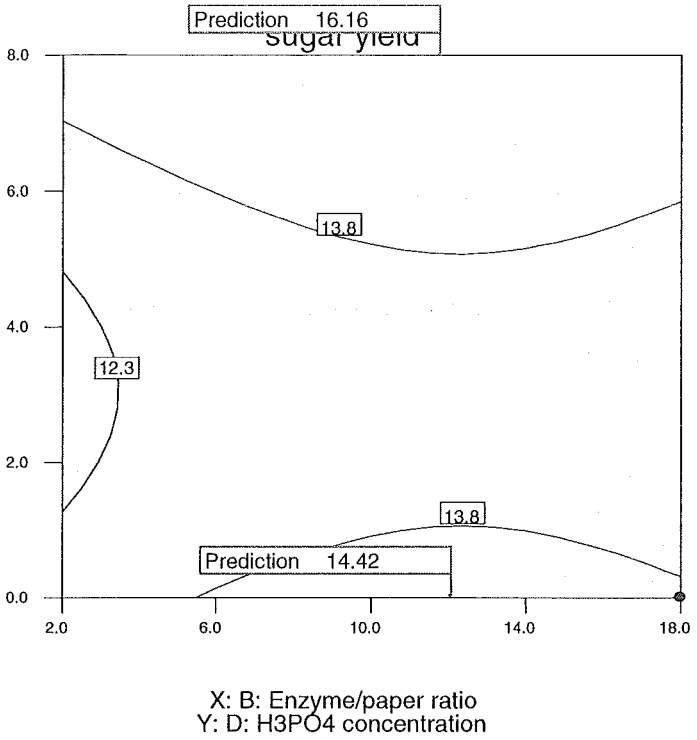


Figure 4.27 (a) Response interaction graph between enzyme/paper ratio and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of enzyme/paper ratio and H_3PO_4 concentration

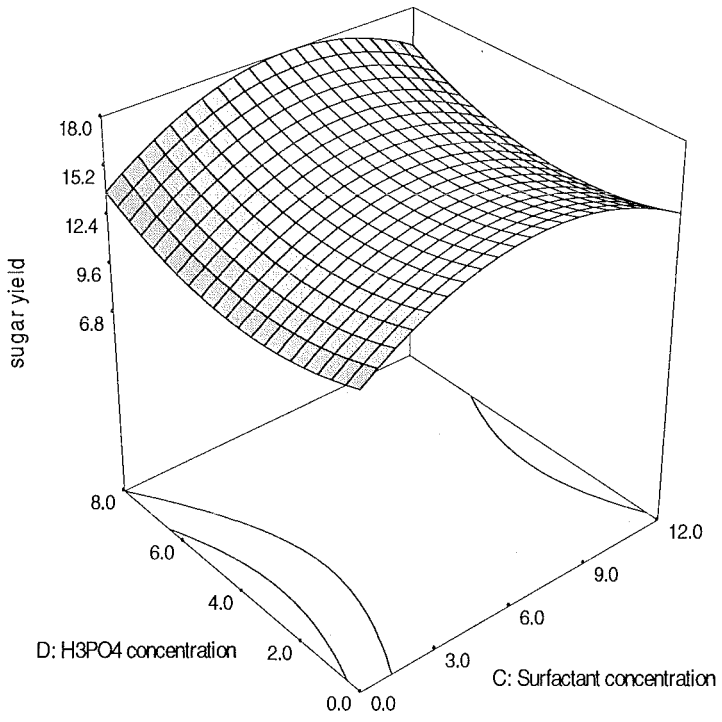


Figure 4.28 Response surface of sugar yield as a function of H_3PO_4 concentration and surfactant concentration at reaction time (20 h) and enzyme/paper ratio (18 %)

The relationship between the extent of H_3PO_4 concentration and surfactant concentration at fixed reaction time (20 h) and enzyme/paper ratio (18 %) is shown in **Figure 4.28**. The figure shows that sugar yield increased with increasing surfactant concentration up to a certain point. Beyond this, the yield started to decrease. With varying H_3PO_4 concentration, the opposite was observed. Sugar yield obtained with samples with no pretreatment and a maximum amount of H_3PO_4 pretreatment samples produced a higher amount of reducing sugars than H_3PO_4 pretreated samples close to 4 g/L. (see **Figure 4.28** and **Figure 4.29 (b)**).

Figure 4.29 (a) and **(b)** illustrates that the maximum sugar yield could be obtained at the highest amount of H_3PO_4 concentration at 8 g/L and surfactant concentration at approximately 6.8 g/L. An 18 % sugar yield was obtained at this point. The difference in saccharification between untreated samples and pretreated samples at 8 g/L, at the lowest and the highest surfactant concentration showed marginal improvements of 9.5 % (see **Equation 4.23**) and 12.1 % (see **Equation 4.24**) respectively.

$$\left(\frac{13.8-12.6}{12.6}\right)\times 100$$

$$= 9.5 \qquad \textbf{(4.23)}$$

$$\left(\frac{15.7-14.0}{14.0}\right)\times 100$$

$$= 12.1 \qquad \textbf{(4.24)}$$

It was clearly seen that pretreatment was required to render cellulose amenable to enzymatic conversion to glucose. Without pretreatment, native cellulose could not be broken down by cellulase to monomer sugars, due to the structural features of the substrate itself. This included lignin content, hemicellulose and the degree of crystallinity. In this case, lignin played a major role in the extent of hydrolysis because removal of lignin could dramatically increase hydrolysis (Sun and Cheng, 2002). Since the pretreatment method used in this study did not successfully remove or reduce lignin content significantly, thus most lignin is still in tight contact with hemicellulose and cellulose. It was probable that hemicellulose was not able to solubilise completely when acid pretreatment was employed and was still cross-linked with lignin. If the lignin barrier was removed, hemicellulose could be easily solubilised because it is amorphous in its natural state (Gan *et al.*, 2003). Nevertheless, pretreatment improved newspaper hydrolysis

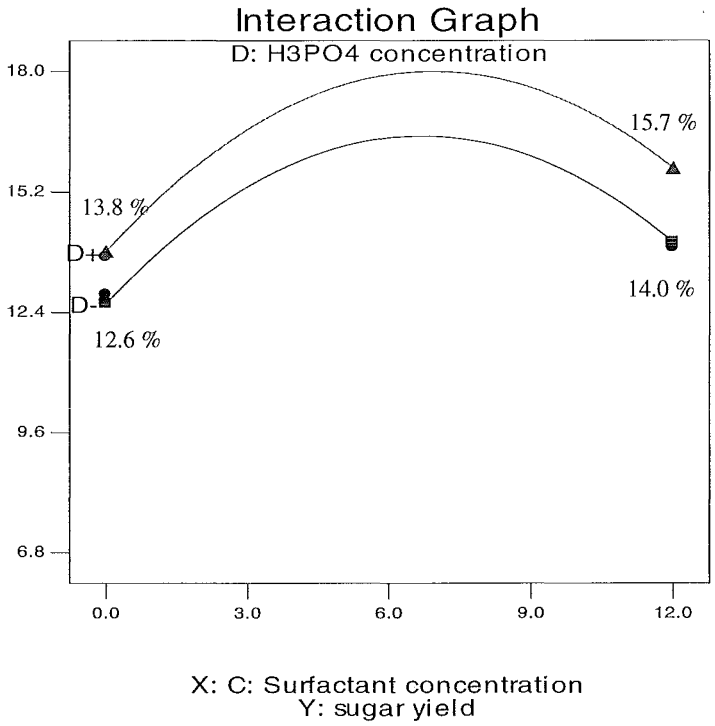
moderately because acid pretreatment could have reduced its crystallinity content and solubilised parts of hemicellulose.

Enzymatic hydrolysis of cellulose involves adsorption of cellulase onto the substrate surface, biodegradation into reducing sugars followed by desorption of cellulase. Without surfactant addition, cellulase would irreversibly bind to the lignin surface and consequently, more cellulase would need to be added in order to increase the hydrolysis rate. As a result, surfactant was employed to prevent irreversible binding of cellulase on cellulose. Increasing surfactant loading clearly enhanced the cellulose hydrolysis for both pretreated and non-pretreated samples. This also indicated that pretreated samples still contained a large amount of lignin after pretreatment because the amount of surfactant concentration was similar to the untreated ones. At maximum surfactant concentration however, a decrease in sugar yield was observed. Deactivation or inadequate cellulase may not be the cause of the decreasing yield, but it was most likely due to the inaccessibility of cellulase to the active binding sites.

The susceptibility of surfactant and cellulase to binding with the substrate depended on its accessibility. Increasing surfactant concentration up to 12 g/L did not provide additional adsorption on lignin for both pretreated samples and non-pretreated samples because the lignin was not exposed to the outer surface and the void spaces in between the microfibrils were too small to be penetrated through and bind with the rest of the lignin. Cellulase also experienced the same situation where most cellulase was not able to reach the entire amorphous surface and the altered crystalline cellulose after pretreatment. This was because the ability of cellulase to reach the available internal surface area of the cellulose was still very limited. Surfactant and cellulase could still be recovered from the liquid supernatant as they could not access further into the internal substrate surface.

With these excessive surfactant and cellulase remaining active in the solution, a longer period of time was essential for continuous degradation of cellulose. After the initial batch of cellulase had fully degraded the amorphous parts of the cellulose, it was expected that more available area and void spaces had been created. The structure of the cellulose would eventually collapse and lignin would redistribute and reveal some weaker spots. This encouraged the rest of the surfactant and cellulase to attach to the lignin and cellulose specifically and therefore, an increase in hydrolysis yield could be observed with an increase in reaction time.

(a)



(b)

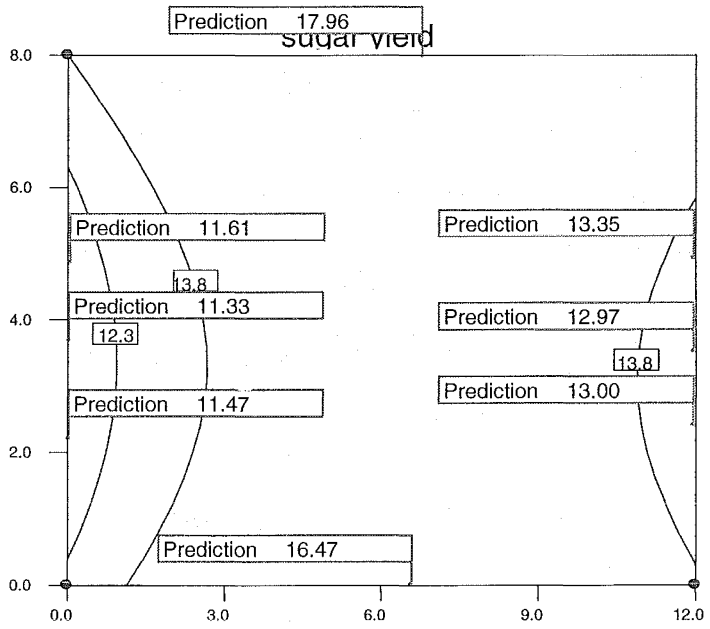


Figure 4.29 (a) Response interaction graph between surfactant concentration and H_3PO_4 concentration at (▲) 8 g/L and (■) 0 g/L and (b) response contours between the interaction of surfactant concentration and H_3PO_4 concentration

4.2.3 Optimisation

Table 4.7 shows the performance of LCRSM compared to a genetic algorithm (refer to **Appendix D**). Both models gave the same maximum sugar yield prediction of 18 %, with similar optimum conditions for the four factors. This was because the three-dimensional surface graphs from *Design Expert Version 6* were comparatively smooth and hence, it was easy to detect the maximum sugar yield. An experiment was conducted to verify the validity of the existence of the optimum point and it was found to be 17.13 %. This meant that the actual response value obtained was in good agreement with the predicted response value.

Table 4.7 Comparison of the optimum four factors and the maximum possible conversion of newspaper between (a) LCRSM and (b) genetic algorithms

(a)

Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Maximum sugar yield (%)
20.0	18.0	6.8	8.0	18.0

(b)

Reaction time (h)	Enzyme/paper ratio (%)	Surfactant (g/L)	H_3PO_4 (g/L)	Maximum sugar yield (%)
20.0	18.0	6.9	8.0	18.0

With the use of LCRSM, there was clear information about the interaction of these four factors in achieving the highest sugar yield. This also demonstrated that the main effects of these factors could be obtained by conducting less experiments compared to other standard response surface methods. This therefore, agreed with the evidence provided by Allen and Yu (2002).

5 CONCLUSIONS

In this study, it was shown that optimisation of reaction time, enzyme/paper ratio, H_3PO_4 pretreatment and surfactant addition using LCRSM could significantly increase overall sugar yield for both office paper and newspaper. The optimum conditions for office paper were obtained as follows: reaction time = 20 hours, enzyme/paper ratio = 18 %, H_3PO_4 = 8 g/L, surfactant = 4.2 g/L and predicted sugar yield = 86.6 %. For newspaper, the optimum conditions were: reaction time = 20 hours, enzyme/paper ratio = 18 %, H_3PO_4 = 8 g/L, surfactant = 6.8 g/L and predicted sugar yield = 18 %. The employment of LCRSM could predict the main effects of the four factors and their interaction on the response quite precisely such that sugar yield for office paper and newspaper obtained experimentally was in fairly good agreement with predicted values, at 82.2 % and 17.13 % respectively, which also verified the existence of an optimum point. Furthermore, the optimum conditions and the predicted sugar yield results obtained for office paper and newspaper were similar to those predicted using Genetic Algorithms (GAs). This indicated that the number of experimental runs could be reduced to save cost, time and effort, which could not be achieved with other standard experimental design methods.

Reaction time was an important factor that allowed cellulase to adsorb on the cellulose surface before degrading them into reducing sugars. Long reaction time periods were crucial for efficient interaction between cellulase and cellulose. Cellulase was another influential factor that acted as a prerequisite for enzymatic hydrolysis of cellulose. The concentration of cellulase would determine the extent of saccharification yield. Since lignocellulosic materials are heterogeneous in nature, they were very resistant to enzymatic attack and therefore, the overall rate of reaction was extremely low. However, the rate of hydrolysis of native cellulose was dependent on its chemical composition. Office paper has low lignin content and a substantially high amount of cellulose, which consisted of both resistant crystalline and amorphous structures. On the other hand, newspaper contained high lignin content and cellulose, together with other structural components such as hemicellulose. Although office paper experienced a higher hydrolysis yield than newspaper, the yield slopes for both substrates showed a decreasing pattern by the end of the reaction time.

In order to improve enzymatic hydrolysis, pretreatment was applied to the native insoluble substrates and increased degradation could be observed with both types of substrates. High H_3PO_4 concentration could be used to improve sugar yield performance. Substrate crystallinity

could have altered, causing a reduction of crystallinity and at the same time, the amorphous portion of cellulose increased. However, the reduction of lignin content during pretreatment was very minimal. Hence, a significant improvement of digestibility was seen with office paper, but not with newspaper. While the digestibility of both substrates increased upon H_3PO_4 pretreatment, substrates that were treated with 4 g/L H_3PO_4 concentration had low sugar yield. The most plausible explanation could be that lignin surfaces had redistributed and recondensed, causing a low conversion of cellulose. As a result, an appropriate pretreatment method should have been chosen to remove lignin and reduce crystalline portions at the same time.

Since substrates are heterogeneous in nature, the rate of hydrolysis was expected to be influenced by the amount of cellulose surface accessible to the cellulase. After pretreatment, there was an increase in accessible surface area and hence, an increase in reaction rate was observed. In other words, correlations existed among substrate structural features, available surface area and the rate of reaction. When the available surface area was increased, more cellulase was able to adsorb onto the cellulose and to further degrade them into reducing sugars. Newspaper however, may not undergo substantial increase of available surface area because large amounts of lignin still remain cross-linked with hemicellulose and cellulose and consequently, most cellulase failed to attach with the entire available cellulose surface. With the addition of moisture, the substrate may swell and aid in improving the enzymatic hydrolysis of high lignin-containing substrate.

During enzymatic hydrolysis, the degree of irreversible adsorption of cellulase was dependent on the amount of lignin exposed on the outer substrate surface. The irreversible binding of cellulase on office paper was less than newspaper because of its low lignin content. Surfactant addition improved the hydrolysis yield by binding onto the hydrophobic lignin surface and allowing cellulase to attach to specific active binding sites. Therefore, the amount of surfactant loading used in office paper was minimal. As for newspaper, control of unspecific adsorption of cellulase to the substrate was improved with surfactant addition up to a certain amount. Beyond that concentration, it was not beneficial to the hydrolysis yield. Excessive amounts of surfactant left in the solution would affect the initial hydrolysis rate by minimising the accessible passageway of cellulase to attach with the substrate surface. It would take some time for the cellulase to successfully pass through a layer of surfactant and further degrade the amount of existing surface area. Therefore, optimum concentration of surfactant was required for efficient enzymatic hydrolysis of lignocellulosics.

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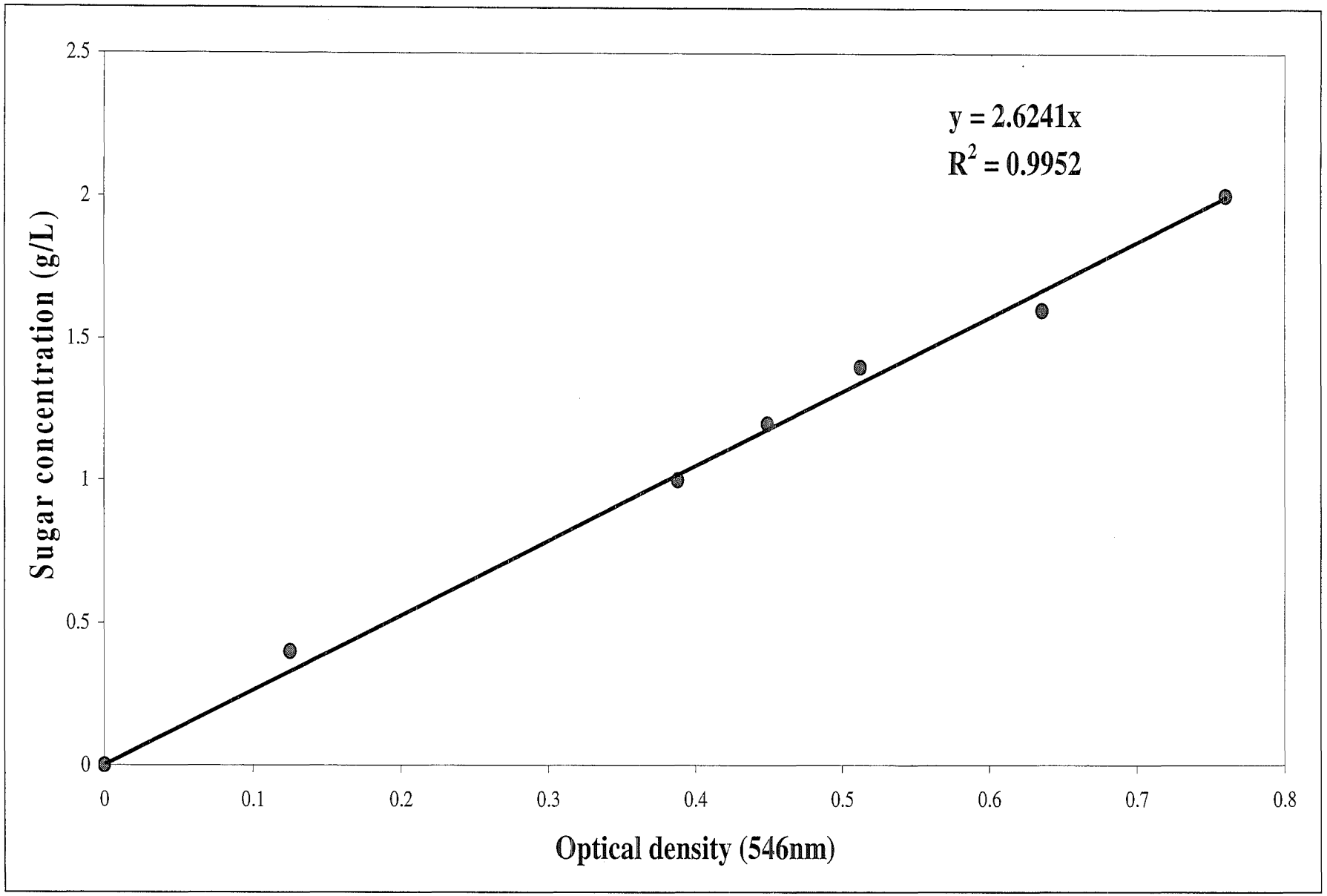

Figure A1 Calibration of DNS reagent using standard glucose solutions 0.0-2.0 g/L

APPENDIX B

Office Paper

Variables	
A (h)	Reaction time
B (%)	Enzyme/paper ratio
C (g/L)	Surfactant concentration
D (g/L)	H_3PO_4 concentration

ANOVA Tables for Reduced Quadratic Models with 14 Runs

Model Form #1

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4424.00	10.00	442.40	51.22	0.004	*significant*
A	1900.49	1.00	1900.49	220.05	0.0007	
B	2397.57	1.00	2397.57	277.61	0.0005	
C	0.31	1.00	0.31	0.036	0.8621	
D	0.074	1.00	0.074	0.008625	0.9319	
A^2	32.24	1.00	32.24	3.73	0.1489	
B^2	234.23	1.00	234.23	27.12	0.0138	
C^2	67.44	1.00	67.44	7.81	0.0682	
AB	0.32	1.00	0.32	0.037	0.8591	
AC	63.09	1.00	63.09	7.30	0.0736	
BC	50.97	1.00	50.97	5.90	0.0934	

(b)

Std. Dev.	2.94	R-Squared	0.9942
Mean	45.73	Adj R-Squared	0.9748
C.V.	6.43	Pred R-Squared	-5.3870
PRESS	28421.37	Adeq Presicion	25.8510

Model Form #2

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4373.20	10.00	437.32	17.10	0.0196	*significant*
A	1867.04	1.00	1867.04	73.02	0.0034	
B	1929.86	1.00	1929.86	75.48	0.0032	
C	0.33	1.00	0.33	0.013	0.9173	
D	0.65	1.00	0.65	0.025	0.8833	
A^2	74.12	1.00	74.12	2.90	0.1872	
B^2	294.16	1.00	294.16	11.50	0.0427	
D^2	17.37	1.00	17.37	0.68	0.4703	
AB	2.35	1.00	2.35	0.092	0.7817	
AD	34.26	1.00	34.26	1.34	0.3308	
BD	62.22	1.00	62.22	2.43	0.2167	

(b)

Std. Dev.	5.06	R-Squared	0.9828
Mean	45.73	Adj R-Squared	0.9253
C.V.	11.06	Pred R-Squared	-17.9773
PRESS	84447.24	Adeq Presicion	15.125

Model Form #3

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4224.93	10.00	422.49	5.63	0.0908	*not significant*
A	1646.11	1.00	1646.11	21.95	0.0184	
B	2105.10	1.00	2105.10	28.07	0.0131	
C	4.93	1.00	4.93	0.07	0.8142	
D	2.57	1.00	2.57	0.03	0.8650	
A^2	114.23	1.00	114.23	1.52	0.3050	
C^2	150.97	1.00	150.97	2.01	0.2510	
D^2	1.33	1.00	1.33	0.02	0.9026	
AC	60.69	1.00	60.69	0.81	0.4346	
AD	95.16	1.00	95.16	1.27	0.3419	
CD	6.48	1.00	6.48	0.09	0.7880	

(b)

Std. Dev.	8.66	R-Squared	0.9494
Mean	45.73	Adj R-Squared	0.7809
C.V.	18.94	Pred R-Squared	-72.4652
PRESS	3.269E+005	Adeq Presicion	8.133

Model Form #4

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4403.30	10.00	440.33	28.34	0.0094	*significant*
A	1567.50	1.00	1567.50	100.90	0.0021	
B	2058.08	1.00	2058.08	132.48	0.0014	
C	0.61	1.00	0.61	0.04	0.8551	
D	2.02	1.00	2.02	0.13	0.7424	
B^2	302.51	1.00	302.51	19.47	0.0216	
C^2	111.03	1.00	111.03	7.15	0.0755	
D^2	22.79	1.00	22.79	1.47	0.3125	
BC	71.26	1.00	71.26	4.59	0.1217	
BD	57.00	1.00	57.00	3.67	0.1513	
CD	0.094	1.00	0.094	0.006082	0.9427	

(b)

Std. Dev.	3.94	R-Squared	0.9895
Mean	45.73	Adj R-Squared	0.9546
C.V.	8.62	Pred R-Squared	-10.5168
PRESS	51248.86	Adeq Presicion	19.732

ANOVA Tables for Reduced Quadratic Models with 18 Runs

Model Form #1

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5817.43	10.00	581.74	15.23	0.0008	*significant*
A	1819.16	1.00	1819.16	47.63	0.0002	
B	2534.90	1.00	2534.90	66.38	<0.0001	
C	8.77	1.00	8.77	0.39	0.6464	
D	26.30	1.00	26.30	0.69	0.4340	
A^2	18.55	1.00	18.55	0.49	0.5083	
B^2	210.38	1.00	210.38	5.51	0.0513	
C^2	27.84	1.00	27.84	0.73	0.4214	
AB	0.93	1.00	0.93	0.024	0.8802	
AC	0.19	1.00	0.19	0.01	0.9456	
BC	5.53	1.00	5.53	0.14	0.7148	

(b)

Std. Dev.	6.18	R-Squared	0.9561
Mean	44.12	Adj R-Squared	0.8933
C.V.	14.01	Pred R-Squared	0.5425
PRESS	2783.86	Adeq Presicion	14.215

Model Form #2

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5992.58	10.00	599.26	45.51	<0.0001	*significant*
A	2328.36	1.00	2328.36	176.83	<0.0001	
B	2666.78	1.00	2666.78	202.53	<0.0001	
C	2.01	1.00	2.01	0.15	0.7078	
D	2.58	1.00	2.58	0.20	0.6716	
A^2	87.09	1.00	87.09	6.61	0.0369	
B^2	304.64	1.00	304.64	23.14	0.0019	
D^2	22.45	1.00	22.45	1.70	0.2329	
AB	4.44	1.00	4.44	0.34	0.5797	
AD	42.92	1.00	42.92	3.26	0.1140	
BD	148.36	1.00	148.36	11.27	0.0121	

(b)

Std. Dev.	3.63	R-Squared	0.9849
Mean	44.12	Adj R-Squared	0.9632
C.V.	8.22	Pred R-Squared	0.8372
PRESS	990.72	Adeq Presicion	24.646

Model Form #3

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5681.64	10.00	568.16	9.87	0.0030	*significant*
A	2334.09	1.00	2334.09	40.53	0.0004	
B	2669.49	1.00	2669.49	46.35	0.0003	
C	10.19	1.00	10.19	0.18	0.6867	
D	48.28	1.00	48.28	0.84	0.3903	
A^2	82.66	1.00	82.66	1.44	0.2699	
C^2	107.32	1.00	107.32	1.86	0.2145	
D^2	0.13	1.00	0.13	0.002260	0.9634	
AC	5.30	1.00	5.30	0.09	0.7705	
AD	84.91	1.00	84.91	1.47	0.2640	
CD	0.22	1.00	0.22	0.003794	0.9526	

(b)

Std. Dev.	7.59	R-Squared	0.9337
Mean	44.12	Adj R-Squared	0.8391
C.V.	17.20	Pred R-Squared	0.3959
PRESS	3675.84	Adeq Presicion	11.728

Model Form #4

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	5975.30	10.00	597.53	38.22	<0.0001	*significant*
A	2503.77	1.00	2503.77	160.09	<0.0001	
B	2515.41	1.00	2515.41	160.88	<0.0001	
C	0.010	1.00	0.010	0.006639	0.9802	
D	1.29	1.00	1.29	0.083	0.7820	
B^2	322.08	1.00	322.08	20.60	0.0027	
C^2	101.59	1.00	101.59	6.50	0.0382	
D^2	24.61	1.00	24.61	1.57	0.2499	
AC	22.61	1.00	22.61	1.45	0.2682	
AD	165.12	1.00	165.12	10.56	0.0141	
CD	9.55	1.00	9.55	0.61	0.4600	

(b)

Std. Dev.	3.95	R-Squared	0.9820
Mean	44.12	Adj R-Squared	0.9563
C.V.	8.96	Pred R-Squared	0.8292
PRESS	1039.34	Adeq Presicion	22.786

ANOVA Table for a Full Quadratic Model with 18 Runs

Full Model Form

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	6080.82	14.00	434.34	331.52	0.0002	*significant*
A	1990.66	1.00	1990.66	1519.40	<0.0001	
B	2405.41	1.00	2405.41	1835.96	<0.0001	
C	0.0009528	1.00	0.0009528	0.0007273	0.9802	
D	0.013	1.00	0.013	0.009877	0.9271	
A^2	48.88	1.00	48.88	37.31	0.0088	
B^2	205.12	1.00	205.12	156.56	0.0011	
C^2	76.19	1.00	76.19	58.16	0.0047	
D^2	38.51	1.00	38.51	29.39	0.0123	
AB	0.54	1.00	0.54	0.41	0.5679	
AC	7.69	1.00	7.69	5.87	0.0939	
AD	47.98	1.00	47.98	36.62	0.0091	
BC	5.57	1.00	5.57	4.25	0.1313	
BD	194.36	1.00	194.36	148.35	0.0012	
CD	2.00	1.00	2.00	1.52	0.3050	

(b)

Std. Dev.	1.14	R-Squared	0.9994
Mean	44.12	Adj R-Squared	0.9963
C.V.	2.59	Pred R-Squared	0.7119
PRESS	1753.07	Adeq Presicion	67.276

APPENDIX C

Newspaper

ANOVA Tables for Reduced Quadratic Models with 14 Runs

Model Form #1

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	114.80	10.00	11.48	5.70	0.0894	*not significant*
A	54.74	1.00	54.74	27.17	0.0137	
B	16.69	1.00	16.69	8.28	0.0636	
C	7.14	1.00	7.14	3.54	0.1563	
D	1.44	1.00	1.44	0.72	0.4598	
A^2	6.01	1.00	6.01	2.98	0.1825	
B^2	0.41	1.00	0.41	0.21	0.6808	
C^2	14.22	1.00	14.22	7.06	0.0766	
AB	1.84	1.00	1.84	0.92	0.4092	
AC	7.18	1.00	7.18	3.56	0.1556	
BC	12.35	1.00	12.35	6.13	0.0896	

(b)

Std. Dev.	1.42	R-Squared	0.9500
Mean	9.32	Adj R-Squared	0.7833
C.V.	15.22	Pred R-Squared	-49.1613
PRESS	6061.63	Adeq Presicion	7.291

Model Form #2

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	107.04	10.00	10.70	2.33	0.2639	*not significant*
A	62.28	1.00	62.28	13.54	0.0348	
B	7.67	1.00	7.67	1.67	0.2873	
C	11.57	1.00	11.57	2.52	0.2110	
D	0.59	1.00	0.59	0.13	0.7449	
A^2	2.00	1.00	2.00	0.44	0.5566	
B^2	4.13	1.00	4.13	0.90	0.4132	
D^2	1.84	1.00	1.84	0.40	0.5721	
AB	0.15	1.00	0.15	0.032	0.8696	
AD	13.39	1.00	13.39	2.91	0.1866	
BD	7.19	1.00	7.19	1.56	0.3000	

(b)

Std. Dev.	2.15	R-Squared	0.8858
Mean	9.32	Adj R-Squared	0.5050
C.V.	23.01	Pred R-Squared	-119.9902
PRESS	14620.79	Adeq Presicion	4.581

Model Form #3

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	119.42	10.00	11.94	25.27	0.0111	*significant*
A	42.86	1.00	42.86	90.68	0.0025	
B	10.26	1.00	10.26	21.70	0.0187	
C	12.12	1.00	12.12	25.65	0.0149	
D	0.55	1.00	0.55	1.17	0.3594	
A^2	2.68	1.00	2.68	5.67	0.0976	
C^2	16.68	1.00	16.68	35.30	0.0095	
D^2	4.26	1.00	4.26	9.01	0.0576	
AC	4.77	1.00	4.77	10.09	0.0502	
AD	17.37	1.00	17.37	36.76	0.0090	
CD	1.39	1.00	1.39	2.93	0.1852	

(b)

Std. Dev.	0.69	R-Squared	0.9883
Mean	9.32	Adj R-Squared	0.9492
C.V.	7.37	Pred R-Squared	-9.4665
PRESS	1264.80	Adeq Presicion	15.043

Model Form #4

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	115.78	10.00	11.58	6.86	0.0700	*not significant*
A	54.68	1.00	54.68	32.38	0.0108	
B	20.84	1.00	20.84	12.34	0.0391	
C	9.57	1.00	9.57	5.67	0.0975	
D	0.48	1.00	0.48	0.29	0.6303	
B^2	1.11	1.00	1.11	0.66	0.4761	
C^2	13.44	1.00	13.44	7.96	0.0667	
D^2	6.24	1.00	6.24	3.69	0.1504	
BC	15.14	1.00	15.14	8.97	0.0579	
BD	4.34	1.00	4.34	2.57	0.2073	
CD	0.12	1.00	0.12	0.069	0.8103	

(b)

Std. Dev.	1.30	R-Squared	0.9581
Mean	9.32	Adj R-Squared	0.8183
C.V.	13.94	Pred R-Squared	-40.2375
PRESS	4983.25	Adeq Presicion	7.197

ANOVA Tables for Reduced Quadratic Models with 18 Runs

Model Form #1

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	167.48	10.00	16.75	15.11	0.0008	*significant*
A	63.05	1.00	63.05	56.88	0.0001	
B	20.99	1.00	20.99	18.93	0.0034	
C	8.08	1.00	8.08	7.29	0.0307	
D	0.50	1.00	0.50	0.45	0.5218	
A^2	6.95	1.00	6.95	6.27	0.0408	
B^2	0.43	1.00	0.43	0.38	0.5553	
C^2	13.48	1.00	13.48	12.16	0.0102	
AB	4.17	1.00	4.17	3.76	0.0937	
AC	10.84	1.00	10.84	9.78	0.0167	
BC	13.35	1.00	13.35	12.04	0.0104	

(b)

Std. Dev.	1.05	R-Squared	0.9557
Mean	8.95	Adj R-Squared	0.8925
C.V.	11.77	Pred R-Squared	-0.0412
PRESS	182.46	Adeq Presicion	13.015

Model Form #2

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	138.46	10.00	13.85	2.63	0.1057	*not significant*
A	77.35	1.00	77.35	14.72	0.0064	
B	12.28	1.00	12.28	2.34	0.1702	
C	0.84	1.00	0.84	0.16	0.7016	
D	3.39	1.00	3.39	0.64	0.4484	
A^2	0.70	1.00	0.70	0.13	0.7268	
B^2	6.11	1.00	6.11	1.16	0.3167	
D^2	1.76	1.00	1.76	0.34	0.5807	
AB	0.79	1.00	0.79	0.15	0.7094	
AD	0.92	1.00	0.92	0.17	0.6887	
BD	1.70	1.00	1.70	0.32	0.5869	

(b)

Std. Dev.	2.29	R-Squared	0.7901
Mean	8.95	Adj R-Squared	0.4902
C.V.	25.62	Pred R-Squared	-0.7042
PRESS	298.64	Adeq Presicion	5.518

Model Form #3

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	156.26	10.00	15.63	5.76	0.0148	*significant*
A	110.07	1.00	110.07	40.60	0.0004	
B	22.87	1.00	22.87	8.43	0.0228	
C	2.22	1.00	2.22	0.82	0.3954	
D	2.08	1.00	2.08	0.77	0.4100	
A^2	0.97	1.00	0.97	0.36	0.5679	
C^2	13.37	1.00	13.37	4.93	0.0618	
D^2	3.37	1.00	3.37	1.24	0.3018	
AC	10.65	1.00	10.65	3.93	0.0879	
AD	3.13	1.00	3.13	1.15	0.3182	
CD	0.026	1.00	0.026	0.009745	0.9241	

(b)

Std. Dev.	1.65	R-Squared	0.8917
Mean	8.95	Adj R-Squared	0.7370
C.V.	18.40	Pred R-Squared	-0.2045
PRESS	211.07	Adeq Presicion	8.315

Model Form #4

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	158.17	10.00	15.82	6.49	0.0106	*significant*
A	84.04	1.00	84.04	34.47	0.0006	
B	12.57	1.00	12.57	5.16	0.0574	
C	3.48	1.00	3.48	1.43	0.2713	
D	0.0000811	1.00	0.0000811	0.00003327	0.9956	
B^2	1.18	1.00	1.18	0.48	0.5098	
C^2	12.74	1.00	12.74	5.23	0.0561	
D^2	10.44	1.00	10.44	4.28	0.0772	
BC	12.82	1.00	12.82	5.26	0.0556	
BD	0.006339	1.00	0.006339	0.0026	0.9608	
CD	0.035	1.00	0.035	0.014	0.9083	

(b)

Std. Dev.	1.56	R-Squared	0.9026
Mean	8.95	Adj R-Squared	0.7635
C.V.	17.45	Pred R-Squared	-0.9164
PRESS	335.82	Adeq Presicion	9.075

ANOVA Table for a Full Quadratic Model with 18 Runs

Full Model Form

(a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	174.34	14.00	12.45	41.41	0.0053	*significant*
A	64.70	1.00	64.70	215.15	0.0007	
B	14.27	1.00	14.27	47.45	0.0063	
C	9.64	1.00	9.64	32.06	0.0109	
D	0.89	1.00	0.89	2.95	0.1842	
A^2	4.72	1.00	4.72	15.71	0.0287	
B^2	1.24	1.00	1.24	4.12	0.1354	
C^2	15.92	1.00	15.92	52.95	0.0054	
D^2	5.68	1.00	5.68	18.88	0.0225	
AB	2.40	1.00	2.40	7.97	0.0665	
AC	9.81	1.00	9.81	32.61	0.0107	
AD	1.09	1.00	1.09	3.63	0.1527	
BC	14.80	1.00	14.80	49.21	0.0059	
BD	0.002431	1.00	0.002431	0.008083	0.9340	
CD	0.12	1.00	0.12	0.39	0.5783	

(b)

Std. Dev.	0.55	R-Squared	0.9949
Mean	8.95	Adj R-Squared	0.9708
C.V.	6.13	Pred R-Squared	0.0610
PRESS	164.56	Adeq Presicion	20.306

APPENDIX D

Office Paper

MATLAB Function File for Office Paper Full Model

```
function [sol, val] = gaDemo1Eval(sol,options)
% Demonstration evaluation function used in gademo1.
%
% function [val,sol] = gaDemo1Eval(sol,options)
%
% val - the fittness of this individual
% sol - the individual, returned to allow for Lamarckian
evolution
% options - [current_generation]
%
% >> initPop=initializega(100,[4 20;2 18;0 8;0 8],
'eval_lowcost_full_model');
% >> [x endPop] = ga([4 20;2 18;0 8;0 8],
'eval_lowcost_full_model',[],initPop,[1e-6 1 1],
'maxGenTerm',100,...
%   'normGeomSelect',[0.08],['arithXover'],[2 0]);

x1=sol(1);
x2=sol(2);
x3=sol(3);
x4=sol(4);

val = - 13.18078 + 3.74549*x1 + 5.09555*x2 + 3.45562*x3 -
5.00201*x4 - 0.085623*x1^2 - 0.16832*x2^2 - 0.42761*x3^2 +
0.30399*x4^2 - 4.50395e-003*x1*x2 + 0.032609*x1*x3 +
0.081437*x1*x4 - 0.029029*x2*x3 + 0.17150*x2*x4 -
0.033219*x3*x4;
```

Newspaper

MATLAB Function File for Newspaper full Model

```
function [sol, val] = gaDemo1Eval(sol,options)
% Demonstration evaluation function used in gademo1.
%
% function [val,sol] = gaDemo1Eval(sol,options)
%
% val - the fittness of this individual
% sol - the individual, returned to allow for Lamarckian
evolution
% options - [current_generation]
%
% >> initPop=initializega(100,[4 20;2 18;0 12;0 8],
'eval_newspaper_full_model');
% >> [x endPop] = ga([4 20;2 18;0 12;0 8],
'eval_newspaper_full_model',[],initPop,[1e-6 1 1],
'maxGenTerm',100,...
%   'normGeomSelect',[0.08],['arithXover'],[2 0]);

x1=sol(1);
x2=sol(2);
x3=sol(3);
x4=sol(4);

val = + 4.58552 - 0.55155*x1 + 0.51196*x2 + 1.23714*x3 -
1.01835*x4 + 0.026615*x1^2 - 0.013081*x2^2 - 0.086879*x3^2 +
0.11671*x4^2 + 9.52488E-003*x1*x2 + 0.024547*x1*x3 +
0.012289*x1*x4 - 0.031550*x2*x3 -6.06489E-004*x2*x4 + 5.34323E-
003*x3*x4;
```